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Characteristics of the RNA Synthesized *in vitro* by Human Erythroblasts[1]

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Several metabolic features of the erythroid differentiation have been recently clarified It has been shown that the synthetic rates of both RNA and protein decrease during maturation, although at a different rate [3, 15] Reticulocytes are able to actively synthesize protein, whereas they no longer synthesize RNA [6] RNA of reticulocytes is synthesized in an early precursor cell and remains functional through the reticulocyte stage [2] Maturation from erythroblast to reticulocyte is characterized by a decrease in number and size of polyribosomes [9]

An important achievement in recent years has been the demonstration that in animal cells ribosomal RNA is not synthesized directly in its final form, but through a complex molecular sequence Evidence obtained in different cell lines, such as HeLa cells [8, 11, 18] and cultured lymphocytes [16], suggests that such a sequence in RNA metabolism is a general feature of human cells However, up to the present this has not been shown in human erythroblasts

It is at present beyond technical possibilities to obtain from human bone marrow erythroblastic populations at a definite stage of maturation and free from large contamination by other cell types Therefore, *we have attempted to obtain information concerning RNA synthesis* in human erythroblasts through the study of the nucleated red cells present in the peripheral blood in cases of hemolytic anemia and idiopatic myelofibrosis Although such cells cannot be considered identical to normal bone marrow erythroblasts, we believe that the basic features

[1] Research supported by a grant from the Consiglio Nazionale delle Ricerche (Contract No. 115 1848 0 1336)

of their RNA metabolism may be considered as essentially similar to those of normal erythroblasts. We have studied in these cells the kinetics of uridine-^3H incorporation into RNA and the characteristics of sedimentation in sucrose gradients of the RNA labeled after different periods of incubation with uridine-^3H. Sedimentation experiments were also carried out after incubation with methyl-^{14}C-methionine, since it has been shown that methylation of RNA offers an extremely sensitive means to follow the processing of the newly synthesized ribosomal RNA [4, 16].

Material and Methods

Case material. The cells studied were obtained from 3 patients, chosen because of the unusually high counts of their nucleated circulating red cells. Patient 1 was a 9 year-old boy suffering from severe hemolytic anemia since a few months of age. He had been splenectomized at the age of three, but the only change observed after splenectomy was a relevant increase in the number of nucleated circulating red cells. By accurate hematological screening, thalassemia and abnormal hemoglobin syndromes had been excluded. Several enzyme activities had been evaluated, and a markedly reduced level of G6PD activity had been found. Metabolic studies in this patient have been reported in detail elsewhere [11]. At the time of the present investigation, peripheral cell count was as follows: RBC 2,150,000, nucleated red cells 35,000, WBC 9,600, platelets 185,000. In patient 2, a 56-year-old female, myelofibrosis had been diagnosed 3 years before. Conspicuous splenomegaly was present. The patient had been previously treated with splenic irradiation and busulfan, but at present she was only under androgen treatment, which had started 6 months previously. Peripheral count was as follows: RBC 2,500,000, nucleated red cells 28,000, WBC 11,000, platelets 125,000. In patient 3, a 47-year-old male, anemia and myelofibrosis had been found 4 years before. He had been splenectomized, but the anemia had not improved significantly. When our studies were performed, the peripheral pattern was as follows: RBC 2,250,000, nucleated red cells 31,000, WBC 13,500, platelets 285,000.

Preparation of erythroblasts. 30 ml of heparinized blood (20 U/ml) were filtered slowly through a column of commercial nylon fibers (Leukopak, Fenwal Lab., Morton Grove, Ill.), 10 × 100 mm, maintained at 37°C. The filtered blood was then sedimented by gravity at 37°C and the supernatant plasma was discarded. The erythrocyte layer, containing the erythroblasts, was diluted with minimal essential medium (MEM) to a concentration of 1–2 × 10^9 erythrocytes per ml. Erythroblast concentrations ranged from 2 to 4 × 10^7 per ml. Five ml fractions were distributed in 25 ml rubber stoppered test tubes rotating at 37°C, in an atmosphere of 95% O_2 to 5% CO_2. All operations were performed under sterile conditions.

Time course studies. To study the kinetics of incorporation of labeled uridine into RNA, the radioactive precursor (uridine-5-^3H, 16.0 c/mmole, Radiochemical Centre, Amersham, England) was added to a 5 ml culture, at the final concentration of 10 μc/ml. 200 μl samples were removed at different intervals, pipetted into 5 ml of 5% TCA at 4°C and held at this temperature for at least 1 h. Incorporation of labeled precursor into DNA was minimal, as indicated by the low radioactivity of DNA isolated by the MARMUR [7] procedure after incubation with uridine-^3H. RNA radioactivity was assayed on the precipitate by the modification of the SCHNEIDER [13] procedure already described [16].

cessed as above mentioned.

Sucrose gradient analysis For sedimentation analysis of uridine-^{3}H labeled RNA, aliquots of the labeled cultures were harvested at intervals of 30 min, 45 min, 1, 4, 6 and 12 h of exposure to the label For sedimentation analysis of RNA labeled after incubation of erythroblasts with methyl-^{14}C-methionine, 10 μc of the radioactive precursor (Radiochemical Centre, 46 0 mc/mmole) were added to 1 ml cultures prepared in methionine-free medium, which were harvested after 30, 60 and 120 min of incubation

Chicago Corp, Des Plaines, Ill)

Results

Characteristics of the erythroblastic populations Nucleated cells in our cultures were represented almost exclusively by erythroblasts. Leukocytes were less than 1%. The distribution of the different maturation stages, the percentages of cells labeled with thymidine-^{3}H and uridine-^{3}H and the average grain counts in the three populations studied are shown in table I The characteristics of the erythroblastic population in case 1 were definitely different from those of cases 2 and 3, which on the contrary were quite similar. In case 1, virtually all the erythroblasts were at the orthochromatic stage, i e. the cytoplasm did not show any basophilia and the chromatin of the nucleus was greatly condensed. When studied by autoradiography none of them incorporated thymidine-^{3}H and only a few showed some labeling with uridine-

Table I. Distribution of different maturation stages and average labeling indexes in the 3 erythroblastic populations studied. 1,000 erythroblasts were counted in each case

Case No.	Maturation stage	Percent of cells	Thymidine-^{3}H		Uridine-^{3}H	
			Percent of labeled cells	Average grain count	Percent of labeled cells	Average grain count
1	baso	0	–	–	–	–
	poly	0.5	0	–	100	19
	ortho	99.5	0	–	9	8
2	baso	0	–	–	–	–
	poly	18	12	21	100	45
	ortho	82	0	–	25	18
3	baso	0	–	–	–	–
	poly	21	9	15	100	28
	ortho	79	0	–	19	16

^{3}H. However, since liquid scintillation counting indicated that a definite amount of radioactive precursor was incorporated into the RNA of these cells in a 30 min period, it seems justified to suppose that a fairly higher percentage of nucleated red cells took up some label, but this amount remained beyond the limits of sensitivity of the autoradiographic method. In cases 2 and 3, on the other hand, a fair portion of the erythroblastic population was at the polychromatic stage, i.e. the cytoplasm showed a varying degree of basophilia and the chromatin was irregularly lumped. Some cells at this stage were labeled after incubation with thymidine-^{3}H, and all of them were heavily labeled with uridine-^{3}H. Only a small portion of the orthochromatic erythroblasts showed autoradiographically some uptake of uridine-^{3}H.

The observation that, after 48 h of incubation, there was a marked reduction in the number of cells of cases 2 and 3 showing cytoplasmic basophilia, as well as in the number of cells labeled with uridine-^{3}H, may indicate that some degree of 'maturation' occurred during the incubation period.

Time course of uridine-^{3}H incorporation. Circulating human erythroblasts maintained *in vitro* in a synthetic medium incorporated uridine-^{3}H into RNA for at least 18 h. As shown in figure 1, the amount of acid insoluble radioactive material increased for about 6 h in case 1,

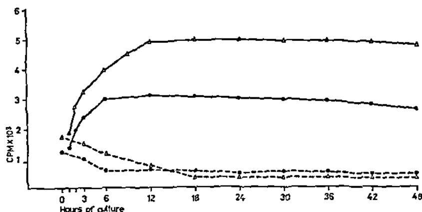
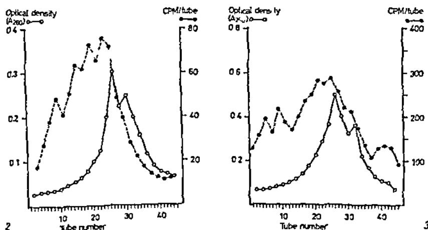


Fig 1 Kinetics of uridine-^3H incorporation by circulating human erythroblasts. The cpm on the ordinate represent the total acid insoluble radioactive material in 200 μl samples [illegible]



[illegible] pattern of labeled RNA extracted [illegible]

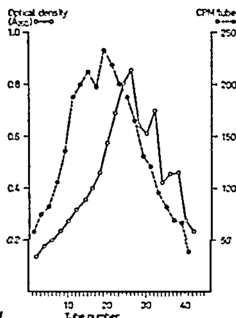
sucrose

then reached a plateau decreasing very slowly over the next 10 h. In case 2, on the other hand, this amount continued to increase over the first 12 h, reaching a plateau after this interval.

The amount of radioactivity incorporated in a 30 min interval diminished by increasing the period of preincubation in the absence of the precursor, but remained approximately constant after the 6th hour in case 1 and after the 12th hour in case 2.

When cultures previously incubated with uridine-^{3}H for 45 min were added with actinomycin D at the concentration of 5 μg/ml, about 85% of the radioactivity became acid soluble in a period of three hours. (At the concentration of 5 μg/ml, actinomycin D completely prevented incorporation of uridine-^{3}H into RNA.)

These results suggest that the time course of incorporation in our experiments reflected both synthesis and decay. In the first period of erythroblast survival *in vitro*, new production appeared to exceed



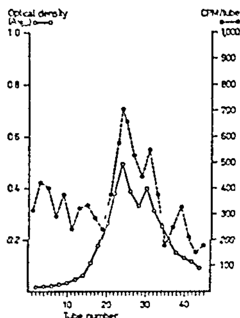


Fig. 4. Sedimentation pattern of labeled RNA from erythroblasts of case 2 incubated with uridine-^{3}H (10 μc/ml) for 45 min. Centrifugation was at 16,000 rpm for 16 h at 4°C, in 5–20% sucrose.

Fig. 5. Sedimentation pattern of labeled RNA from erythroblasts of case 2 incubated with uridine-^{3}H (10 μc/ml) for 6 h. Centrifugation at 16,000 rpm for 16 h at 4°C, in 5–20% sucrose.

breakdown, and there was a net increase in the radioactivity incorporated by the cells exposed to the labeled precursor After an interval of 12 h in cases 2 but of only 6 h in case 1, the rate of RNA synthesis decreased to a level at which it could only equal breakdown The behavior of cells from case 3 was quite similar to that of cells from case 2

Sedimentation patterns of labeled RNA after incubation with uridine ³H The sedimentation pattern of whole cell RNA extracted from circulating erythroblasts of patient 1, incubated with uridine ³H for 45 min, is shown in figure 2 Most of the radioactivity was distributed in the region of the gradient corresponding to sedimentation coefficients of 30–60 S or more After 6 h of incubation of the same cells, the bulk of radioactive RNA partially overlapped the region of 28 S and 18 S RNA components although the sedimentation profiles clearly indi

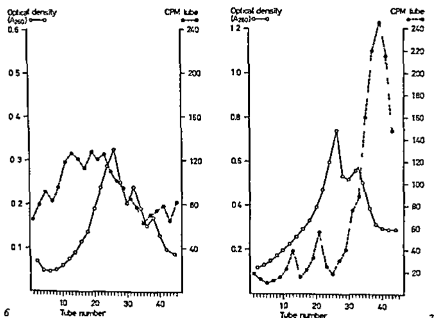


Fig 6 Sedimentation pattern of labeled RNA from erythroblasts of case 2 incubated with uridine-³H (10 µc/ml) for 6 h Centrifugation at 16 000 rpm for 16 h at 4°C, in 5–20% sucrose

Fig 7 Sedimentation pattern of labeled RNA from erythroblasts of case 1 incubated with methyl ¹⁴C-methionine (10 µc/ml) for 2 h. Centrifugation at 18 000 rpm for 16 h at 4°C, in 5–20% sucrose

cated that most of the radioactivity sedimented faster than ribosomal RNA (fig. 3). When the same cells were exposed for 6 h to uridine-^{3}H after 24 h of preincubation without the labeled precursor, most of the radioactivity sedimented with RNA showing S values higher than 30.

When erythroblasts from case 2, as well as from case 3, were incubated with uridine-^{3}H for 45 min, the sedimentation patterns observed were quite similar to that shown in case 1, i.e. most of the radioactivity sedimented in the 30–60 S region of the gradient (fig. 4). On the contrary, when the same cells were incubated with labeled uridine for 6 h, 3 definite radioactive peaks were observed which coincided with the optical density peaks of 28, 18 and 4 S (fig. 5). The pattern was only slightly different when the cells were incubated for 6 h following a 12-hour period of preincubation without the labeled precursor, but when the preincubation lasted for 24 h, only a small portion of radioactivity sedimented with ribosomal RNA (fig. 6), and the pattern resembled that observed with cells from case 1.

Sedimentation patterns of labeled RNA after incubation of erythroblasts with methyl-^{14}C-methionine. The result of the sucrose gradient analysis of

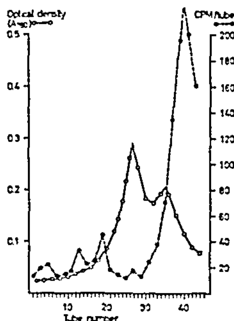


Fig. 8. Sedimentation pattern of labeled RNA from erythroblasts of case 2 incubated with methyl-^{14}C-methionine (10 μc/ml) for 30 min. Centrifugation at 18,000 rpm for 16 h at 4 °C, in 5–20 sucrose.

RNA extracted from the erythroblasts of case 1, incubated with methyl-^{14}C-methionine for 2 h is shown in figure 7. Besides a fairly large amount of radioactivity in the 4 S region, presumably representing methylated soluble RNA and methionyl sRNA, two peaks of radioactivity were observed in the region of the gradient between 32 and 45 S (fig. 7). In erythroblasts from cases 2 and 3, after the same period of incubation, the 28 S ribosomal RNA peak was definitely labeled whereas a pattern similar to that obtained with cells from case 1, i.e. with radioactive peaks between 32 and 45 S, was observed after only 30 min of incubation with methyl ^{14}C-methionine (fig. 8).

Discussion

In all the erythroblastic populations studied the newly synthesized RNA consisted mainly of molecules sedimenting faster than ribosomal RNA, with S values ranging from 30–70 S. After 6 h of incubation with labeled uridine, however, the pattern in case 1 was fairly different from that observed in cases 2 and 3. In the former case only a little radioactivity was associated with ribosomal RNA, whereas in the erythroblastic populations of cases 2 and 3, a relevant proportion of the label sedimented with ribosomal and transfer RNA. Since actinomycin D caused a high rate of RNA breakdown in circulating erythroblasts, possibly by preventing the metabolic stabilization of the newly formed molecules, it was not possible to follow by actinomycin 'chase' the fate of the RNA labeled after short term incubation. However, our experiments show in human erythroblasts, after incubation with methyl-^{14}C-methionine, the presence of methylated RNA fractions having S values ranging from 32–45 S. This therefore suggests that the rapidly sedimenting RNA labeled with uridine-^{3}H in short term incubation of erythroid cells must be considered, at least in part, a ribosomal precursor [4].

In erythroblasts from case 1, on the other hand, uridine ^{3}H experiments failed to show any definite labeling of ribosomal RNA after several hours of incubation with the precursor. These cells therefore showed clearly the same seeming discrepancy between the results obtained with uridine-^{3}H and those obtained with methyl-^{14}C-methionine which has been already observed in small lymphocytes [16]. We presume that also in mature erythroblasts the high specificity of methyl

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Sedimentation patterns of labeled RNA after incubation of erythroblasts with methyl ¹⁴C methionine The result of the sucrose gradient analysis of

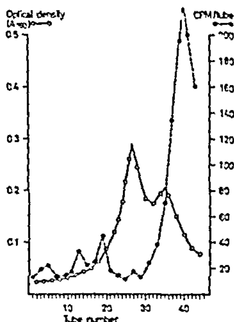


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labeling allows detection of the synthesis of small amounts of ribosomal RNA which in uridine ^{3}H experiments are overshadowed by larger amounts of nonribosomal RNA.

Since cell populations from patients with different diseases are considered, one might speculate that the variations observed are related to the different pathological conditions examined. However, it seems likely that the different results are rather due to the different maturation levels of the cell populations studied. In cases 2 and 3, in fact, a fair proportion of the cells was at the polychromatic stage, whereas in case 1 the cell population was formed only by mature orthochromatic erythroblasts.

Under our conditions of cell maintenance *in vitro*, rate of RNA synthesis decreased in all populations studied, reaching a plateau after an interval which in case 1 was much shorter than in cases 2 and 3, and sedimentation experiments indicate that the decrease concerned mainly ribosomal RNA. Since a population containing only mature cells synthesize much less ribosomal RNA than a population containing immature cells, it seems reasonable to suppose that the decrease in ribosomal RNA synthesis which accompanies the *in vitro* survival is not the result of *in vitro* conditions, but rather may be associated with the maturation process.

Our results do not give any information about the functional significance of the rapidly sedimenting non ribosomal RNA synthesized in human mature erythroblasts. The synthesis of an RNA with similar characteristics has been already observed in several types of cells, such as duck nucleated erythrocytes [1, 12], HeLa cells [17] and human small lymphocytes [16].

It seems possible to conclude that erythroblastic populations formed by cells at different level of maturation synthesize a high molecular weight RNA which is, at least in part, a ribosomal precursor. The synthesis of ribosomal precursor RNA decreases during the incubation of erythroblasts *in vitro*. Rather than being the result of *in vitro* conditions, this phenomenon, as previously implied, could be related to the maturation of erythroblasts. In fact, the rate of synthesis of ribosomal precursor was much lower in one of the populations studied formed only by mature orthochromatic erythroblasts.

Acknowledgement. We wish to thank Mr. E. [illegible] for his generous financial help and Dr. Mario [illegible] of Research Laboratories Farmitalia, Milan, for gifts of KB cells.

Summary

Erythroblastic populations of different maturation levels synthesize a high molecular

stud ed, formed only by mature orthochromatic cells

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Reticulocyte Generation Time and Rate of Discharge from the Marrow [1]

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The number of reticulocytes in the circulating blood is regarded as a valid index of erythropoietic activity Since practically all red cells are discharged from the marrow while they still posses varying amounts of the substantia granulo filamentosa (SGF) [1], the commencement of the reticulocytosis may also be used as an index of red cell release into the circulation, particularly when erythropoiesis is newly established after a period of depression Furthermore, the amount of SGF is inversely proportional to the stage of maturity of reticulocytes [2], and therefore, the maturation time of red cell precursors will be represented by the time required for the youngest forms of reticulocytes to appear in the marrow and blood following an erythropoietic stimulus A quantitative assessment of the reticulocyte response will indicate not only the time required for maturation of red cell precursors, but will also permit a more accurate estimation of the numbers of cells which entered on erythroid differentiation in response to the stimulus

The polycythemic animal in which erythropoiesis is depressed and in which there are few reticulocytes in either marrow or blood, provides a convenient starting point for the study of red cell maturation following an erythropoietic stimulus

[1] Supported in part by NIH grant No AM CA 13145 to C Rosse and in part by research funds of the Department of Anatomy University of Bristol England

Materials and Methods

Animals and Procedure. Polycythemia (around 6×10^6 mm³ blood) was induced in young male guinea pigs of Dunklin-Hartley strain by subjecting them to hypoxia in a chamber at ½ atmospheric pressure. After 7 days of hypoxia, the guinea pigs were kept in ambient air (period of 'rebound'). On the 9th day of rebound they were divided into two groups: in one group, 10–12 ml of blood was withdrawn by cardiac puncture under light ether anesthesia, in the second group, the animals were not bled and they served as the control. The bled animals were killed in groups of 8 on days, 1, 1½, 2, 2½, 3, 4, 5 and 6 after bleeding. The control animals were also sacrificed in groups of 8 at daily intervals for 6 days (i.e., on the 9th to 15th days of rebound).

Red cell count, hemoglobin, PCV estimations and reticulocyte studies were carried out on mixed venous and arterial blood obtained from the aorta and inferior vena cava at the time of exsanguination. The spleen was weighed and fixed in Zenker formol. Sections were cut at 2 μ and stained with a modification of Dominici's stain.

Reticulocyte counts. Marrow and blood reticulocytes were studied on fixed smears [3]. The number of reticulocytes per mm³ of blood was determined from the red cell count, and their number per mm³ of marrow from the nucleated cell count obtained by the quantitative technique of YOFFEY [4].

Differential counts of reticulocytes were also made in both blood and marrow, classifying 200 reticulocytes in each into four maturation stages [2] as illustrated in figure 1.

Statistical analysis. Means and standard deviations were calculated and the data were compared using Student's 't' test. Changes were regarded as significant if $'p' < 0.05$ and highly significant if $'p' < 0.001$.

Results

Red cell count. In the unbled controls the polycythemia was maintained until the 12th day of rebound, but on the 13th day a significant decrease occurred representing a return toward normal values [5]. In bled animals, the red cell count remained significantly below normal to the end of the observation period ($2.72 \pm 0.56 \rightarrow 3.90 \pm 0.68 \times 10^6$/mm³). Hemoglobin and PCV paralleled the changes in the red cell count.

The spleen of bled animals showed the first signs of weight increase on the 3rd day ($612 \pm 105 \rightarrow 917 \pm 254$ mg). This was accompanied by the appearance of young erythroblasts. No change was observed in the controls.

The Quantitative Reticulocyte Response

a) Marrow reticulocytes (fig. 2). In the marrow of the controls the number of reticulocytes remained constant at a level markedly below the normal [6]. In bled animals, however, a significant fall was



Fig 1 Reticulocytes at different maturation grades in the blood, 4 days after bleeding. Grade I the youngest forms contain a compact mass of SGF, almost completely filling the cell. In grade II the SGF is looser. In grade III it is in the form of loose filaments and granules. Grade IV the most mature forms contain only a few scattered granules. Cells containing a nucleus as well as SGF were not classified as reticulocytes. Marked anisocytosis is present. Stain new methylene blue supravitally. MacNeal's tetrachrome counterstain × 1 800

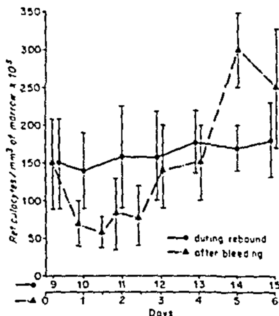


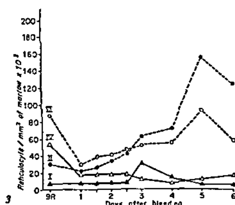
Fig. 2. Number of reticulocytes in the marrow during rebound and after bleeding. Mean ± SD.

observed 24 h after bleeding ($t = 2.5$; $0.05 > p > 0.02$), representing the first measurable effect of the stimulus. Although an increase became evident on the 3rd day, only on the 5th day did it reach a significant level ($t = 10.7$; $p < 0.001$).

b) Blood reticulocytes. In the blood of the controls, reticulocytes remained at a level significantly below normal until the 14th day of rebound, when a threefold increase ($t = 2.8$; $p < 0.01$) restored their level to normal values [1]. Although in bled animals the values showed a wide variation, the first increase clearly occurred from day 2½ to day 3.

Reticulocytes of Different Maturation Grades

a) Marrow (fig. 3). After bleeding the initial fall in reticulocytes was due to a significant decrease in the more mature forms. The youngest reticulocytes (grade I) showed the first increase from day 2½ to day 3 ($t = 2.8$, $0.05 > p > 0.02$), but this increase was not maintained. The most mature forms (grade IV) did not show any increase throughout the experiment.

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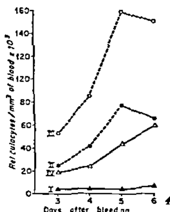
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Fig 3 Reticulocytes of different maturation grades in the marrow after bleeding Mean values

Fig 4 Reticulocytes of different maturation grades in the blood after bleeding Mean values.

b) Blood (fig 4) Up to the 3rd day, reticulocytes were so few in the blood that their accurate grading was not possible They were predominantly of the more mature type On the 3rd day, however, reticulocytes of all grades appeared in the blood, and, even grade I continued to be present throughout the experiment Grade IV showed a continuous increase, reaching a highly significant rise by the 6th day ($t = 6\ 5$, $p < 0\ 001$) In unbled animals the onset of peripheral reticulocytosis was associated with the appearance of grade I reticulocytes in blood and marrow on the 14th day of rebound

Discussion

Reticulocyte discharge from the marrow In unstimulated animals the abrupt increase in blood reticulocytes was preceded by the return of the red cell count to the range of normal Although the peripheral reticulocytosis was heralded by the reappearance of erythroblasts [7], restoration of the marrow reticulocyte reserve to the normal level did not take place In stimulated animals, however, in contrast to the controls, a highly significant accumulation of reticulocytes within the

marrow paralleled the rise in blood reticulocytes Comparison of these observations with previous studies suggests that the accumulation of reticulocytes in the marrow may be related to the severity of the erythropoietic stimulus No increase was noted in the marrow at simulated altitudes of 10 000 feet [8] 14 000 feet [5, 9], but a marked increase occurred at 17,000 [10] and 20,000 [8] During stimulation the increase in marrow reticulocytes was not due to the blocking of the discharge mechanism as it had been suggested [11] The following observations indicate that reticulocyte discharge was, in fact, accelerated while their numbers were rising within the marrow (a) marrow and blood reticulocytes increased simultaneously, (b) the peripheral reticulocytosis was unlikely to be due to a splenic contribution, since the splenic response lagged behind that of the marrow, and also because erythropoietin does not produce a reticulocyte release from the spleen, as it does from the marrow [12], (c) grade I reticulocytes showed only a transient peak in the marrow, but they were continuously present in the blood following that peak They are usually absent from normal blood, (d) in contrast to normal guinea pig blood, grade II and grade III reticulocytes outnumbered the most mature forms, and (e) grade IV reticulocytes never accumulated in the marrow, but showed a continuous increase in the blood

The uncorrected anemia and the similarity of the reticulocyte curve to the response obtained by repeated daily injections of erythropoietin [13] suggest that in the present experiment the stimulus was operating continuously Consequently, the accumulation of reticulocytes in the marrow, in spite of their accelerated discharge, can only be explained by a faster rate of red cell production than red cell release

The timing of the reticulocyte response The latent period before an increase in circulating reticulocytes following erythropoietic stimulation of normal marrow is well documented [5, 9, 14] In erythropoietically suppressed marrow this period may be expected to be longer

The non significant increase in more mature forms of marrow reticulocytes on day $1\frac{1}{2}$–$2\frac{1}{2}$ probably reflected the maturation of the few remaining erythroblasts [7] The significant increase in the youngest forms from day $2\frac{1}{2}$ to day 3 on the other hand, must represent the cells that started their maturation from the erythroid precursor cell Since erythropoietin has been shown to be present in blood one hour after bleeding [15] and also to produce a measurable response after acting for 6 h on suppressed marrow [16] the present findings indicate

that the time required for the erythropoietin sensitive cell to reach the reticulocyte stage is of the order of 2½–3 days

Acknowledgement The technical assistance of Mr M Fisher is acknowledged.

Summary

The reticulocyte response was studied in the blood and bone marrow of guinea pig whose erythropoiesis, following prolonged depression was restimulated by massive acute hemorrhage The number of reticulocytes fell in blood and marrow 24 h after the stimulus In the marrow the first increase was noted in the youngest forms of reticulocytes from day 2½ to day 3, indicating the maturation time of red cell precursors from the erythroid [illegible]

reticulocytes

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Pre-Leukaemic Acute Myelogenous Leukaemia[1]

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Kaplan *et al* [8] reported that, in radiationinduced mouse leukaemia treatment of animals with cortisone, in the phase preceding the development of leukaemia, reduced its incidence This hightened the possibility of recognition of a pre-leukaemic state in human leukaemia with far reaching therapeutic implications In 1953, Block *et al.* [3] described 12 patients with anaemia, leukopenia and/or purpura in whom, during the period of the blood dyscrasia lasting from 3 to 17 months, none of the recognizable signs and symptoms of leukaemia were present Later 10 of these patients developed acute myelogenous leukaemia A number of other observers have also reported the development of acute myeloid leukaemia in patients with an initial blood picture of anaemia, neutropenia or thrombocytopenia or various combinations of these lasting for variable periods [1, 2, 5, 7, 10, 12, 13] Whether or not these initial blood changes observed before the onset of recognizable acute leukaemia represent a pre-leukaemic stage in its evolution cannot be definitely stated unless more is known about the nature of leukaemia The purpose of the present communication is to record 7 more instances of acute myelogenous leukaemia in whom a variety of haematologic disorders were encountered before frank leukaemia became evident

Material

The 7 cases reported varied in age from 10 to 58 years. Four were females (table I) They were drawn from a large number of patients referred to our laboratory for blood

[1] Paper presented at the IVth Congress of Asian and Pacific Society of Haematology held in New Delhi in November 1967

Table I Summary of clinical data on 7 patients

Case No.	Age years	Sex	Chief complaints	Pre-leukaemic changes: Duration months	Pre-leukaemic changes: Blood	Bone marrow	Final diagnosis
1	26	M	bony pain, fever	6.5	anaemia, neutropenia	hypercellular, preponderance of myeloid elements	acute myelogenous leukaemia
2	28	M	fever, joint pains	9	anaemia, neutropenia	hypercellular, preponderance of myeloid elements	acute myelogenous leukaemia
3	10	M	purpura	7	thrombocytopenia, neutropenia, anaemia[1]	hypercellular, preponderance of myeloid elements	acute myelogenous leukaemia
4	46	F	weakness, exertional dyspnoea	6	pancytopenia	aplastic	acute myelogenous leukaemia
5	42	F	easy fatiguability, episodes of fever	8	pancytopenia	aplastic	acute myelogenous leukaemia
6	58	F	loss of stamina, dyspnoea	8	anaemia, neutropenia	hypoplastic	acute myelogenous leukaemia
7[1]	32	F	exertional dyspnoea, headaches	18	pancytopenia	aplastic	acute myelogenous leukaemia

[1] History of ingestion of chloramphenicol

examinations during the period 1955 to 1967. Cases 1, 2 and 3 were essentially diagnostic problems and came repeatedly as their blood changes did not conform to any known blood disorder. Many biochemical and bacteriological examinations, omitted from case reports that follow, were performed on these patients to exclude systemic diseases. The other 4 patients were initially diagnosed as suffering from aplastic anaemia and came up for routine

[illegible]

The development of leukaemia, when it occurred, was extremely sudden and the patients rapidly succumbed within 4 to 12 weeks of the onset of the disease

Case Reports

Case 1 A 26-year-old medical graduate complained of bouts of pain around the right hip since December 1955 Earlier, in 1941 he was confined to bed for nearly a fortnight

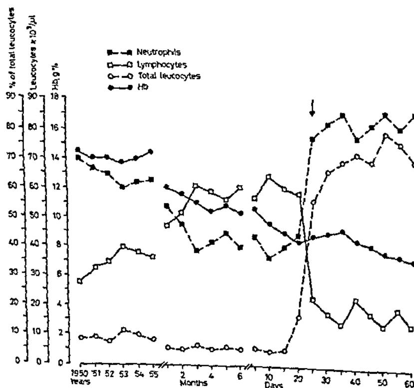


Fig 1 Case 1 blood changes throughout the illness The arrow marks the first appearance of myeloblasts in peripheral blood From this period onwards the graph indicates the percentage of neutrophils together with immature myeloid cells

because of pain all over the body which responded to analgesics, and in 1950 he suffered from two attacks of infectious hepatitis The bony pain was accompanied by episodes of fever (temperature 100–101°F) off and on

Physical examination revealed no positive finding except a localized area of tenderness on the right ileum X-rays of the hip and the chest revealed no abnormality The heamatologic work up of the patient from 1950 to 1955 and all through the period of his illness is set out in figure 1

For nearly 6½ months the patient suffered from progressive, unexplained anaemia and neutropenia During this period the platelets ranged between 170,000–300 000 per µl and the blood smears at no time revealed any abnormal cells The anaemia was normocytic and slightly hypochromic. Bone marrow smears were examined twice and showed hyper-cellularity with an increase in the myeloid elements (table II)

Twenty five days before his death the patient suddenly developed purpuric spots on his limbs, glands became enlarged in the axilla, and the spleen and liver became palpable. Total leukocytes shot up to 78,000/µl, consisting mostly of myeloblasts and promyelocytes.

Case 2 This 28-years-old male sought medical advice several times during the last 9 months of his life because of fever off and on and pain around the joints First his left elbow was involved, a week later the right elbow became affected This was followed 3 months later by swelling around the left ankle The right ankle was similarly affected 2 months after There was no effusion in any of the joints and no limitation of movement except that due to pain The pain was relieved with analgesics

The patient was well nourished, vital signs were normal and the physical examination did not reveal any abnormality X rays of the joints and chest were found normal

A month prior to his death, the patient came in with marked pallor and puffiness around the face Tonsils were not enlarged but enlarged lymphnodes were discernable in the submandibular, submental and posterior cervical regions Gums were swollen and hypertrophic

Table II Cases 1 and 2 differential count of the bone marrow (%)

Cells	Case 1		Case 2		
	1 month[1]	4 months[1]	2 months[1]	5 months[1]	8 months[1]
Myeloblast	2.0	6.6	3.5	5.8	5.0
Promyelocyte	12.5	22.5	11.0	18.6	21.6
Myelocyte	26.0	26.8	23.0	28.2	30.0
Metamyelocyte	19.0	17.0	17.0	7.0	8.5
Neutrophil	14.0	5.0	15.8	12.0	9.0
Eosinophil	3.0	2.0	2.2	–	1.3
Basophil	–	0.5	–	0.6	0.5
Lymphocyte	12.0	6.0	14.6	10.0	8.6
Monocyte	1.5	1.6	2.4	5.0	3.5
Plasma cell	1.0	1.4	–	0.8	1.0
Normoblasts	9.0	10.6	10.5	12.0	8.0
Megakaryocytes	adequate	adequate	adequate	adequate	adequate
M:E ratio	4.5:1	7.5:1	7.0:1	6:1	9.8:1

[1] After the first onset of symptoms

There were a few petechiae and resolving ecchymoses above the left knee A number of hard, discrete, subcutaneous nodules were palpable all over the body, particularly marked on the thighs These were histologically found to be leukaemic deposits The spleen and liver were enlarged 8 cm and 4 cm respectively

The haematologic work up of this patient during the entire period of his illness is charted in figure 2 The significant features were a progressive fall of haemoglobin from 13.8 to 5 5 g% over a period of 9 months preceding the onset of leukaemic symptoms The anaemia was normocytic and hypochromic, associated with neutropenia but the platelets were within normal limits except terminally Prior to the recognition of leukaemia at no time were immature cells seen in peripheral blood Only stray nucleated red cells were found

Bone marrow smears were examined at second, fifth and eighth months after the onset of his symptoms, mainly to exclude a hypoplastic change of the bone marrow The findings presented in table II showed each time hypercellular marrow with an increase in earlier forms of myeloid cells

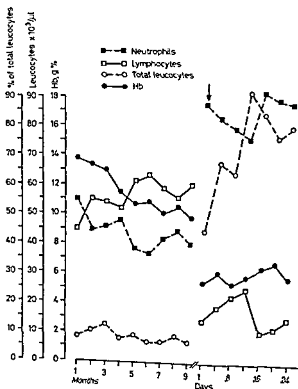


Fig 2 Case 2 blood changes throughout the illness. The arrow marks the first appearance of myeloblasts in peripheral blood. From this period onwards the graph indicates the percentage of neutrophils together with immature myeloid cells.

Case 3 A 10-year-old boy was followed up from March 1955 to July 1956, at which time he came up for his last admission with classical signs and symptoms of acute leukaemia, consisting of fever, marked pallor, stomatitis, tender and bleeding gums, enlargement of lymphnodes in the axillary, cervical and inguinal regions, hepato-splenomegaly and pronounced sternal tenderness.

He sought medical advice first, in March 1955, for episodes of epistaxis coming on occasionally since October 1954 Hematologic investigations during the episodes showed thrombocytopenia, with platelet counts varying from 68,000–110,000 per μl (table III), accompanied by increased bleeding time, positive capillary fragility (++) and poor clot retraction A diagnosis of idiopathic thrombocytopenic purpura was made, but as the episodes of epistaxis were self limiting no treatment was given. Bone marrow smears examined during this period showed no abnormality

From November, 1955 his chief complaints were evening rise of temperature varying from 99 0° to 100 0°F and rarely going up to 102°F, together with progressive loss of weight Physical examination was completely normal Numerous laboratory investigations were obtained, including X-ray of the chest, cultures of blood, urine and sputum, serologic tests for typhoid fever and brucellosis and examination of blood and bone marrow smears for parasites. The haematologic data during the course of his illness is summarized in table III.

Table III Case 3 summary of blood and bone marrow changes

Date	Leukocytes x $10^3/\mu$l	Neutrophils % of WBC	Platelets x $10^3/\mu$l	Hb g%	Bone marrow
March 55	12 5	61 0	72 0	11 0	normocellular M/E ratio 5.8 1 megakaryocytes adequ
June 55	8.6	64 0	85 0	10 8	
Aug 55	9 2	60 0	106 0	10.5	
Nov 55	6.8	49 0	110 0	10.5	
Dec 55	5 0	45 0	90 0	9 8	hypercellular M/E ratio 6 9 1 megakaryocytes adequa
Jan 56	5.5	43 0	85 0	9.5	
Feb. 56	6.2	41 0	94 0	9 7	
March 56	5.8	38 0	98 0	9 3	hypercellular M/F ratio 8 1 megakaryocytes normal
April 56	11 0	49 0	102 0	9 0	
May 56	22.5	69 0[1]	72 0	9 0	
June 56	70 0	87 0[2]	68 0	8 2	
July 56	112 0	95 0[2]	60 0	7 5	
Aug 56	94 0	92 0[2]	76 0	7 0	
Sept. 56	105 0	94 0[2]	71 0	6 8	

[1] Stray myeloblasts seen

[2] Large number of myeloblasts and promyelocytes present

The anaemia was hypochromic and microcytic, characterized by the appearance of fair number of late and intermediate normoblasts in the terminal stages The bone marrow revealed an increase in cellularity with preponderance of relatively immature myeloid elements and an M/E ratio of 6.9 1

The patient was treated with broad spectrum antibiotics, anti tubercular drugs and for Kala Azar without any benefit until May 1956, when typical signs and symptoms of acute leukaemia appeared

Cases 4, 5, 6, 7 These 4 cases who were females between the ages 34 to 58 years may be considered together as the course of their illness was essentially similar They all presented with variable symptoms attributable mainly to progressive anaemia These included pallor, weakness, dyspnoea particularly on exertion, and mild oedema Significant clinical findings were the absence of any liver, spleen and lymphnode enlargement and lack of bony tenderness Other systems were entirely normal.

The haemoglobin in these patients varied from 3.5 to 11.2 g % at different periods There was consistent leukopenia and some degree of thrombocytopenia in at least 3 of the cases (table IV) Examination of the blood smear showed that anaemia was normocytic or macrocytic with MCV ranging between 80 to 102 μm^3 The reticulocyte counts varied from 1 6 to 4.5%

In all these patients the diagnosis of aplastic or hypoplastic anaemia was reached on the basis of peripheral cytopenia and examination of sternal aspirate. In 3 instances hypocellularity of the bone marrow was confirmed by biopsy In case 7, the bone marrow aplasia followed the administration of 4 courses of chloramphenicol at intervals of 2–4 weeks, during which she received a total of 20 0 g of the drug for treatment of an eye infection

As shown in table IV, 6 to 18 months following the onset of aplastic anaemia, these patients developed signs and symptoms that eventually became typical of acute leukaemia. The spleen gradually became enlarged, lymphadenopathy developed and in two the liver also became palpable

Table IV Cases 4–7 summary of haematologic data before transition into acute leukaemia

Case No	Hb g%	Leukocytes x 10³/µl	Platelets x 10³/µl	Bone marrow smear	Biopsy	Duration of aplastic phase months
4	4.5–10 8	1 4–5 3	85–178	hypo-cellular	aplastic	6.0
5	6 0–11 2	2 0–5 4	156–227	hypo-cellular	hypoplastic	8 0
6	3.5–9 0	3 0–7.5	77–196	hypo-cellular	not done	10 0
7	5.2–10 0	1 1–4.8	94–184	hypo-cellular	aplastic	18 0

Each determination shows the range of variation observed during the course of illness.

Discussion

The question may be raised whether it is justifiable to differentiate a pre-leukaemic from a leukaemic phase during the illness of a patient who dies of leukaemia. So diverse are the manifestations of leukaemia that the patient could be considered as having leukaemia from the very onset of symptoms. In our cases such differentiation was made possible only on haematological grounds. Thus, the laboratory investigations showed that in none of the patients, in the pre-leukaemic phase, the characteristic enlargement of any of the haemopoietic tissues was demonstrable. On the other hand, 4 of the patients presented with classical features of aplastic anaemia. We have considered these as important criteria to exclude the diagnosis of leukaemia.

The transition to acute myelogenous leukaemia occurred after a variable period of illness during which two types of changes were observed in the blood. In the first 3 cases there was peripheral cytopenia associated with hypercellular marrow, showing an increase in the proportion of immature myeloid cells. The other 4 patients revealed peripheral cytopenia with hypoplasia or aplasia of the bone marrow, without any increase in the primitive cells.

In their studies on behalf of the Atomic Bomb Casuality Commission, MOLONEY and LANGE [15] followed 4 patients for periods of 5 to 37 months before the development of myelogenous leukaemia. During this period, these patients exhibited variable leukocytosis with a tendency to hypercellularity in the bone marrow associated with myeloid immaturity, in 3 of the 4 cases. Anaemia was not a prominent feature.

The occurrence of leukaemia in case 7 is of particular interest in as far as it illustrates that a nonspecific hypersensitivity reaction, like the one caused by chloramphenicol in this instance, may eventually lead to the development of leukaemia. This is further suggested by the case reported by STRAUSS [16] who observed leukaemia to occur following a second vaccination by typhoid and paratyphoid vaccine.

The relationship, if any, between the haematologic changes observed in the pre-leukaemic period to the development of leukaemia, remains obscure. There is, however, evidence to suggest that both aplasia of the haemopoietic tissue as well as the hyperplastic changes in the bone marrow such as described herein, may be regarded as potentially leukaemic disorders. Thus, observations concerning the effect of benzol on the haemopoietic tissue suggest that a range of variation from

complete aplasia of the bone marrow without any evidence of regeneration to a picture closely resembling leukaemia may develop The myelogenous leukaemia which follows chronic benzol poisoning may be superimposed on aplastic anaemia, or agranulocytosis, the bone marrow being severely hypoplastic or hyperplastic with maturation arrest of granulocytes [4, 6 11] Further, in experimental animals, the production of agranulocytosis following the administration of anti leukocytic serum is associated in the bone marrow with maturation arrest of successively more and more primitive stages in the chain of granulocytes [9, 14] lending the marrow a close resemblance to that of leukaemia, although the peripheral blood shows only a picture of agranulocytosis

It appears that the ultimate reaction of the bone marrow to such agents may be determined by several factors The dose and length of exposure to the agent the condition of the affected individual, and the capacity of the marrow tissue to regenerate or, instead, to form 'scar tissue, are some of the factors considered important [17]

It is common experience that blood and bone marrow changes, as described here in the pre leukaemic stage, rarely terminate in leukaemia When and why do these changes acquire a leukaemic propensity is not possible to state on the basis of available knowledge Further studies directed to characterize the pre leukaemic haemopoietic tissue biochemically or genetically may throw light on the relationship of 'pre leukaemic' states with true leukaemia

Summary

Seven cases are described in whom the development of acute myelogenous leukaemia was preceded by a variety of haematologic disorders. In the pre-leukaemic state which lasted from 6 to 18 months these patients showed anaemia neutropenia or pancytopenia. There was no evidence of haemolysis In 3 cases the bone marrow was hypercellular with increase of relatively immature myeloid cells. In the other 4 it was aplastic. In the pre-leukaemic stage none of the patients manifested signs or symptoms characteristic of leukaemia. The development of leukaemia was sudden and resulted in death within 4 to 12 weeks The problem of pre leukaemic state in the evolution of acute human leukaemia is discussed

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Eosinophil Leucocytes and Recovery from Severe Hypoxia

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In a quantitative study already reported [8], striking changes were observed in the numbers of eosinophil granulocytes in the blood and bone marrow of guinea pigs following continous exposure to a barometric pressure equal to that at an altitude of 20,000 ft (6,096 m) for periods of between 1 and 12 days From the first day of this exposure to severe hypoxia, the blood eosinophils were increased in number, while the marrow eosinophils showed marked depletion This confirmed the findings of a preliminary investigation [16] in which similar changes were noted at a simulated altitude of 20,000 ft (6,096 m) although not at one of 10,000 ft (3,048 m) It was apparent that further investigation would be needed to throw light on the import of these changes and it was hoped that further clues might be provided as to the function of the eosinophil leucocytes In the experiments to be reported here, quantitative methods were used to study the changes in guinea pigs at intervals of between 0 and 7 days following a period of 5 days exposure to severe hypoxia at a simulated altitude of 20,000 ft

Material and Methods

Healthy male albino guinea pigs of the Dunkin-Hartley strain and aged about 6 or 7 weeks old were placed in a decompression chamber and exposed to a barometric pressure (approx. 350 mm Hg) corresponding to that at an altitude of approximately 20 000 ft (6 096 m) for 5 days. The exposure was continous except for a short period each day when the chamber was returned to atmospheric pressure to enable the animals to be weighed, the chamber cleaned and the food and drinking water renewed. The processes of compression and decompression each took about 10 min the rate never being allowed to exceed 4 000 ft (1,220 m) per minute.

Table II. Total nucleated cells and [illegible] subgroups in bone marrow ([illegible] per [illegible])

	Total nucleated	[illegible]	Erythroid	Lympho-cytes	Mono-cytes	Damaged	Other
[illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]
[illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	11 ± [illegible]
[illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± 1
[illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]
[illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]
[illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]
[illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]
[illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]	[illegible] ± [illegible]

[illegible] see table I.

Discussion

The findings in the blood and bone marrow at day 0, immediately after 7 days' exposure to severe hypoxia, were very similar to those reported earlier [3], the main difference being that the blood eosinophil levels were even more strikingly elevated in the present series. In both series, all the marrow granulocytes showed a depression in numbers, the eosinophils being particularly affected and showing evidence of depression at all the three developmental stages studied. The findings at later intervals after the return to normal atmospheric pressure were characterised in general by a return to values similar to those in the control animals. In the previous experiments [3], when the hypoxia was continued for a further period of 7 days (up to a total of 12 days' hypoxia), it was noted that the blood and bone marrow picture continued to be similar to that noted here at day 0. It is concluded from this that the changes observed in the present series between day 0 and

Table III Granulocyte series in bone marrow (thousands per μl)

	Total eosino	Eosino PMC+MC	Eosino MMC	Eosino B+S	Eosino other	Total baso	Total neutr
Control	58 7 ±16 3	5 4 ±3 6	21.2 ±6 5	31.2 ±13 3	0 9 ±0 7	11 1 ±5 7	493 ±14
Day 0	*22 3*[2] ±7 7	2 5 ±1 1	*8 1*[2] ±3 1	*10 8*[3] ±5.5	0 9 ±0.5	7.5 ±1 8	*337*[2] ±88
Day 1	*25 3*[2] ±5 1	2.5 ±1 4	*5 9*[2] ±2 1	*15 7*[3] ±3 6	1 2 ±0 6	7 7 ±4 4	*320*[3] ±60
Day 2	*27 9*[2] ±12 5	2 6 ±1 7	*6 9*[2] ±1 9	17.5 ±10 4	0.9 ±0.3	7 6 ±1 8	417 ±97
Day 3	*23 9*[2] ±6 3	4 6 ±1 3	*8 5*[3] ±4 1	*10 4*[2] ±3 0	0 4 ±0.3	7.2 ±3 1	371 ±73
Day 4	*28 4*[2] ±8 1	3 8 ±1 6	*13 1*[3] ±5 8	*10 9*[3] ±4 4	0 6 ±0 6	7 1 ±3 9	419 ±11
Day 5	*34 3*[2] ±13 9	4 5 ±2 3	17 1 ±8.2	*12 3*[3] ±5 6	0 4 ±0 5	10 2 ±2 9	536 ±11
Day 7	49 7 ±10 3	4 4 ±2 3	16 9 ±3 7	27.3 ±9 3	1 1 ±1 0	14 0 ±6 5	477 ±93

PMC+MC=Promyelocytes and myelocytes. MMC=metamyelocytes. B+S=band and segment forms

[1]Total neutrophils includes myeloblasts

[2] [3] see table I

day 7 were related to the return to normal barometric pressure and can be interpreted as representing the processes of recovery

The results appear to indicate that marrow eosinophil numbers were restored from the stem-cell end of the series When compared with the corresponding control value, significant differences could no longer be detected at day 3 in the case of the promyelocytes and myelocytes, at day 5 with the metamyelocytes, and at day 7 in the case of the band and segmented forms Such a pattern of recovery might be expected if it were the result of increased or restored proliferative activity, a few days being needed for the promyelocytes to develop into mature or almost mature forms A similar pattern of recovery has been observed in the marrow gran [illegible]
administration [illegible]
esis [8] that d[illegible]
the bone marrow had been markedly depressed

From table I, it may be seen that the blood eosinophilia had disappeared by day 2 and that on days 3, 4 and 5 eosinopenia was present. This indicates that in the early stages of recovery, more eosinophils were disappearing from the circulation than were entering it. It was previously noted [8] that during severe hypoxia, the circulatory eosinophilia was associated with a 'shift to the right' in the degree of nuclear segmentation and this was thought consistent with either the eosinophil granulocytes remaining longer in circulation or their re-entering it to a significant degree. The present findings would be consistent with a reversal of this, namely that during recovery there was a more rapid turnover of eosinophils in the blood. Thus, a possible explanation of the eosinopenia on days 3–5 is that it was caused by the time-lag between the commencement of increased eosinophil production in the marrow, and the supply of sufficient numbers of mature forms to maintain the normal blood level. The normal circulation transit time of eosinophil granulocytes is thought to be less than 24 h [3, 5, 14].

Mention may be made here of a preliminary experiment [GRANT, SIBLEY, MOFFATT and HUDSON, unpublished observation] in which a 400 g guinea pig received 5 intraperitoneal injections, each of 1 μC/g of body weight at 2 hourly intervals. After the last injection, it was exposed to severe hypoxia under the same conditions as in the present series for a period of 15 days. Blood smears were made at 12 hourly intervals and 100 eosinophils were examined in autoradiographic preparations at each interval. Unlike a normal animal in which [illegible]

(which was also observed on the 6th day). Although no significance should be attached to this result unless it can be repeated in a larger series, an observation that 100% labelling was not achieved might be explicable in terms of eosinophils remaining longer in circulation or re-entering it after they had left. An early appearance of labelled cells in the blood would not be inconsistent with the changes reported in the early stages of hypoxia [8].

To what extent do the present observations throw light upon factors involved in the eosinophil changes in severe hypoxia? Perhaps one may assume that changes in eosinophil production and distribution merely reflect on underlying disturbances of regulatory mechanisms and in relation to this one may first consider adrenocortical activity. Other quantitative studies [13] have suggested that in certain circumstances at least, cortical hormones may interfere with eosinophilotactic mechanisms, and a similar interpretation can be placed on the observations of other investigators [1, 27]. Furthermore, there is evidence that the adrenal cortex has an important role in adaptation to lowered oxygen tension [28, 29] and it seems likely that the marked increase in adrenal

weight at day 0 (table I) indicated hyperactivity of the adrenal cortex Others have reported both an increase in adrenal weight and a decrease in the size of the thymus [29, 33] during exposure of experimental animals to lowered barometric pressures The adrenal weight has been stated to be a good index of adrenocortical activity in experiments of more than a few days duration [25] although some of the weight increase in severe hypoxia might be attributable to the greatly increased vascularity which is known to occur in this condition [10, 28] In the present experiments, the adrenal glands were still heavier than in the controls for up to 5 days after the period of hypoxia The reduction in size of the eosinophil population of the bone marrow observed at day 0 was rather similar to that seen after the prolonged administration of glucocorticoids or ACTH [2, 6, 7, 19, 31] but the eosinophilia in the blood was in striking contrast to the eosinopenia characteristically seen after cortical hormone administration or in the stress reaction [4] It should be borne in mind that the changes induced in other situations by stress and adrenocortical activity are sometimes difficult to interpret e g in the 'general adaptation syndrome' [25, 26]

Other factors may have been operative here In particular, lowered oxygen tension (or one of the associated physicochemical changes) acting at the cellular level might conceivably have played a part by interfering with enzymatic or other mechanisms

It has already been suggested by other investigations [9, 33] that the general decrease in granulocyte numbers which is observed in the bone marrow of the guinea pig in severe hypoxia (or after haemorrhage) may be partly attributed to the increased commitment of stem cells to red cell production The possibility that local influences (physical, chemical or both) might be at work within the bone marrow has also been discussed [8] but it is thought unlikely that physical growth pressure of the expanding erythroblastic series was very important in the present circumstances The possibility that the pattern of eosinophil cell production in extramedullary sites might differ in severe hypoxia from that in the bone marrow was not excluded by the preliminary observations on the histology of the spleen, although the apparent increase in the number of eosinophils in the spleen on day 0 could have been largely the result of infiltration

The mechanisms underlying normal granulocyte production are known to be complex [23, 24] and perhaps it is not surprising that our understanding of the eosinophil changes in severe hypoxia and subse-

From table I, it may be seen that the blood eosinophilia had disappeared by day 2 and that on days 3, 4 and 5 eosinopenia was present. This indicates that in the early stages of recovery, more eosinophils were disappearing from the circulation than were entering it. It was previously noted [illegible] that during severe hypoxia, the circulatory eosinophilia was associated with a "shift to the right" in the degree of nuclear segmentation and this was thought consistent with either the eosinophil granulocytes remaining longer in circulation or their re-entering it to a significant degree. The present findings would be consistent with a reversal of this pattern that during recovery there was a more rapid removal of eosinophils in the blood. Thus, a possible explanation of the eosinopenia on days 3–5 is that it was caused by the time-lag between the commencement of increased eosinophil production in the marrow, and the arrival of sufficient numbers of mature forms to maintain the normal blood level. The normal circulation transit time of eosinophil granulocytes is thought to be less than 24 h [illegible].

[illegible]

To what extent do the present observations throw light upon factors involved in the eosinophil changes in severe hypoxia? Perhaps we may assume that changes in eosinophil production and distribution merely reflect an unknown disturbance of regulatory mechanisms and in relation to this one may first consider adrenocortical activity. Other comparative studies [illegible] have suggested that in certain circumstances at least, cortical hormones may interfere with eosinophil-forming mechanisms, and a similar interpretation can be placed on the observations of other investigators [illegible]. Furthermore, there is evidence that the adrenal cortex has an important role in adaptation to lowered oxygen tension [illegible] and it seems likely that the marked increase in adrenal

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Haemoglobin O Indonesia (α 116 Glu → Lys) in an Italian Family

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In a preliminary paper some of us [1] presented the results of an investigation on a slow moving haemoglobin which was abnormal in its α-chains.

Case Report

The carrier was a 3-year-old girl admitted to the Paediatric Department of Galliera Hospitals (Italy) with an infectious disease. Before that time she had never suffered from anaemia or severe diseases. The abnormal haemoglobin was detected during a screening of all the patients admitted to the Department. It was impossible to study the parents, but the maternal grandmother, of Sicilian extraction, was found to be a carrier of the same abnormality (fig. 1).

Haematological findings were normal and investigations of the abnormal haemoglobin carried out by means of agar gel electrophoresis at acid pH and starch gel electrophoresis (fig. 2–3) showed the presence of a slow moving fraction amounting to about 20% of the total pigment. The variant haemoglobin which migrated a little slower than Hb S was abnormal in its α-chains as shown by the presence, in concentrated solutions of haemoglobin, of a double A_2 in starch gel (fig. 4) and a double F in agar gel electrophoresis. Further [illegible]

β VIII–IX. No further analyses were possible at that time, but the properties of this haemoglobin variant, compared with others with the same electrophoretic mobility led us to believe it was a new variant which was provisionally called Hb Olivieri from the name of the patient.

The same blood samples were recently re-examined and further analyses were carried out to characterize the amino-acid substitution of this haemoglobin.

[1] On leave from the University Institute of Medical Pathology, Turin, Italy.

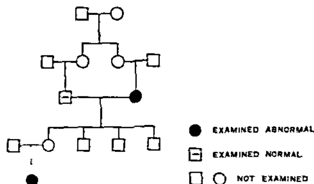


Fig. 1 Family tree



Fig. 2 Starch gel electrophoresis (tris buffer, pH 8.9) of the haemoglobin solution of the propositus (bottom) compared with a normal subject (top). At this haemoglobin concentration the abnormal fraction is clearly separated from Hb A, while the double Hb A_2 is hardly visible

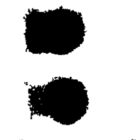


Fig. 3 Starch gel electrophoresis (tris buffer, pH 8.9) of the haemoglobin solution of the propositus (bottom) compared with Hb A–S control (top). The abnormal fraction is slightly slower than Hb S

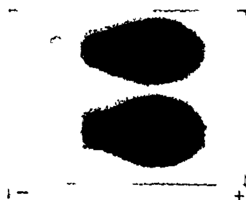


Fig. 4. Starch gel electrophoresis (tris buffer, pH 8.9) of the haemoglobin solution of the propositus (top) compared with a normal control (bottom). The gel was overloaded to show the double Hb A_2.

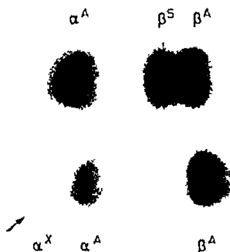


Fig. 5. Cellulose acetate electrophoresis (Urea veronal buffer, pH 8.9) of the globin of the propositus (arrow) compared with the globin of a heterozygote for haemoglobins A and S.

Methods

Haemolysates were prepared from washed red cells and analysed by agar [2] and starch gel electrophoresis [3]. Electrophoresis of the globin in 6 M urea was carried out according to CHERNOFF slightly modified [4]. Hybridization was done following the technique described by HUISMAN *et al.* [5]. The haemoglobin variant was purified by repeated paper electrophoresis in tris buffer pH 8.9 [6] followed by elution and concentration *in vacuo* until it was free from haemoglobin A. Two dimensional peptide maps (fingerprints) of the whole globin and of the aminoethylated chains prepared according to CLEGG *et al.* [7] were obtained using the techniques recently summarized by SICK *et al.* [8]. The tryptic peptides were eluted from the paper with 6 N HCl into capillary tubes and hydrolysed at 108 °C for 20 h. Amino-acids were estimated on an [illegible] automatic analyzer.

Results

Electrophoresis of the globin in 6 M urea [4] and hybridization [5] of the variant haemoglobin with canine haemoglobin showed that the β-chains were normal and that the difference responsible for the reduced electrophoretic mobility resided in the α chains.

Figure 6 shows a fingerprint at pH 6.4 of the variant haemoglobin. A slight increase in the intensity of ninhydrin staining was seen in the region of β Tp VIII IX which represents residues 66–82 of the 146 residues of the β chains. All the other peptides were in their normal positions and gave normal staining reactions. As the α chains were responsible for the abnormality and all peptides belonging to those chains were in their expected positions, the increased intensity in the region of β Tp VIII–IX was thought to be due to a peptide arising from the 'core' of the α-chains which overlapped with β Tp VIII–IX. To confirm this hypothesis, chain separation, aminoethylation of the globin [7] and fingerprinting of the isolated chains was carried out (figs 7 and 8). On aminoethylation (A/E), the 'core' peptides become soluble and are represented on the fingerprint. Even then, in the A/E α chain a small amount of undissolved material is present and while the residues α 100–104 (α Tp XIIa) are well defined, the residues α 105–127 (α Tp XIIb) are found as a poorly defined smear at the

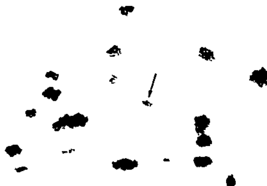


Fig. 6. Peptide chromatogram (fingerprint) of haemoglobin O Indonesia. The arrow indicates the increase in the intensity of ninhydrin staining in the region of β Tp VIII IX.

origin of the fingerprint. When the tryptic digestion of A/E α-chain of the variant haemoglobin was carried out, no insoluble material was seen and the fingerprint (fig. 8) differed from that of A/E α-chain of haemoglobin A by the absence of α Tp XIIb and the presence of two new peptides: one next to α Tp VI which corresponded to the spot with increased intensity of ninhydrin staining in the fingerprint of the

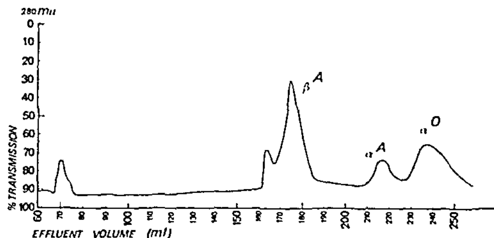


Fig. 7. Chromatography of globin from haemoglobin O Indonesia (40 mg) on CM-cellulose in 8 M urea 2-mercaptoethanol buffers according to Clegg et al. [7]. The gradient was started after the elution volume of 100 ml.

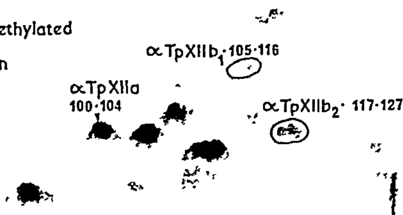


Fig. 8. Peptide chromatogram of the amino-ethylated α chain of haemoglobin O Indonesia. α Tp XIIa is in its expected position, two new peptides are present: α Tp XIIb₁ and α Tp XIIb₂.

whole globin, and the second between α Tp V and α Tp VI Both peptides gave a positive reaction for histidine α Tp XIIa and all the other peptides of the A/E α chain were in their expected position

The new peptides were eluted from a preparative fingerprint, hydrolysed with 6 N HCl for 20 h at 108°C and aminoacid analysis was carried out Table I shows the aminoacid sequence and the point

Table I Amino acid composition of α Tp XIIb of haemoglobin A and O Indonesia (α Tp XIIb comprises residues 105–127 of the 141 residues of the α-chain [7])

	105										115
Hb A	Leu	Leu	Val	Thr	Leu	Ala	Ala	His	Leu	Pro	Ala
	105										115
Hb O	Leu	Leu	Val	Thr	Leu	Ala	Ala	His	Leu	Pro	Ala
	116										127
Hb A	Glu	Phe	Thr	Pro	Ala	Val	His	Ala	Ser	Leu	Asp	Lys
	116										127
Hb O	Lys↑	Phe	Thr	Pro	Ala	Val	His	Ala	Ser	Leu	Asp	Lys

The arrow shows the point of tryptic cleavage in haemoglobin O

Table II Amino acid composition of α Tp $XIIb_1$ and α Tp $XIIb_2$ of Hb O Indonesia

Amino-acid	α Tp $XIIb_1$			α Tp $XIIb_2$		
	μmoles	Molar ratio	Corrected residues	μmoles	Molar ratio	Corrected residues
Aspartic acid	0.004	(0.3)	0	0.014	1.1	1
Threonine	0.018	1.4	1	0.014	1.1	1
Serine	0.008	(0.6)	0	0.014	1.1	1
Glutamic acid	0.003	(0.2)	0	–	–	0
Proline	0.013	1.0	1	0.013	1.0	1
Glycine	0.005	(0.4)	0	–		0
Alanine	0.036	2.8	3	0.024	1.9	2
Valine	0.016	1.2	1	0.012	0.9	1
Leucine	0.050	3.9	4	0.013	1.0	1
Phenylalanine	0.006	(0.5)	0	0.011	0.9	1
Lysine[1]	0.016	1.0	1	0.056	1.0	1
Histidine	0.016	1.0	1	0.062	1.1	1

[1] Basic amino acids were determined on a separate sample and the yields in μmoles per residues are different from the yields for the acidic and neutral amino acids The residues in brackets were assumed to be from a contaminant

Table III. Amino acid composition of α Tp XIIb of Hb A[1] and Hb O Indonesia

Amino acid	No. of residues	
	α Tp XIIb of Hb A	α Tp XIIb$_1$–XIIb$_2$ of Hb O
Aspartic acid	1	1
Threonine	2	2
Serine	1	1
Glutamic acid	1	–
Proline	2	2
Alanine	5	5
Valine	2	2
Leucine	5	5
Phenylalanine	1	1
Lysine	1	2
Histidine	2	2

[1] HILL and KONIGSBERG, 1962 [9].

of tryptic cleavage of the peptide α Tp XIIb (α 105–127) [7]. Table II shows the results of the aminoacid analysis of the 2 new peptides present in the fingerprint of the abnormal haemoglobin. Considered together (table III) they differ from α Tp XIIb of haemoglobin A in the presence of two residues of lysine instead of one and in the absence of glutamic acid. In α Tp XIIb of haemoglobin A glutamic acid is the 116th residue, it is absent in this variant and a lysine is present instead. With the exception of this lysine, the aminoacid composition of α Tp XIIb$_1$ is that of the first 12 aminoacids of α Tp XIIb of haemoglobin A. As the peptide is a tryptic peptide lysine must be the C terminal aminoacid, i.e. is the last residue of the sequence of α Tp XIIb$_1$ (α 105–116). Moreover α Tp XIIb$_2$ shows the same composition of the C terminal end of α Tp XIIb in haemoglobin A, i.e. includes the residues 117–127.

Conclusion

The present haemoglobin shows the following mutation: α 116 Glu → Lys. The same mutation has been described before in haemoglobin O Indonesia. Another case of haemoglobin O Indonesia has been observed by BOTHA, BEALE and LEHMANN [unpublished] in a South African family of 'coloured' non Malay origin. It is likely that the

sporadic findings in Indonesia, South Africa and Italy are due to independent mutations

Summary

A slow moving α-chain abnormal haemoglobin found in an Italian family and described previously as 'Hb Olivieri' has now been identified as haemoglobin O Indonesia. The full demonstration of the substitution α 116 (GH 4) glutamic acid → lysine has been possible.

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Zellkinetik der Granulopoese und des Neutrophilensystems bei einem Fall von zyklischer Neutropenie[1]

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Das Krankheitsbild der zyklischen Neutropenie ist gekennzeichnet durch ein autonomes, periodisches Auftreten und Wiederverschwinden von Beschwerden zusammen mit Fieber, Neutropenie und Monozytose. Durch Beobachtungen und Untersuchungen an etwa 50 Fällen konnte Stellung genommen werden zu Fragen wie Geschlechtsverteilung [26, 27], Symptomatik und Krankheitsverlauf [4, 5, 6, 7, 18, 19, 22, 26, 27, 29, 31, 32], Hormonhaushalt [17, 26, 27, 28, 33], Vererbung [11, 16, 20, 23, 27], z. T. auch zu hämatologischen und pathologisch-anatomischen Problemen [15, 21, 24, 25, 26, 27, 28, 30, 34]. Die Frage nach der Ursache der Krankheitssymptome jedoch blieb nahezu ungeklärt.

Es ist Aufgabe dieser Arbeit, das granulozytäre Zellerneuerungssystem bei einer Patientin mit zyklischer Neutropenie quantitativ, morphologisch und durch Anwendung verschiedener Zellmarkierungsmethoden mit Radioisotopen zellkinetisch zu beschreiben, um zur Klärung der Pathogenese dieser Erkrankung beizutragen.

Material und Methoden

Kasuistik. Untersucht wurde eine 53jährige Patientin (H. K., Journal Nr. 13 9267 der Med. Univ.-Klinik Freiburg), die seit dem 50. Lebensjahr unter Beschwerden litt, welche einförmig und regelmässig in 3wöchigen Abständen rezidivierten. Während des Klinik-

[1] Die Untersuchungen wurden durch die Euratom-Verträge «Strahlenhämatologie» Nr. 031-64-1 BIAD und 072-68-1 BIOD unterstützt.

ausstriche aus der Mundspülflüssigkeit. Die luftgetrockneten und in absolutem Methylalkohol fixierten Ausstriche wurden mit dem Kodak AR10 stripping film autoradiographiert, nach einer Expositionszeit von 69 Tagen entwickelt, fixiert und nach Giemsa gefärbt. Pro Zeitpunkt wurden mindestens 500 myeloische Zellen differenziert und die Silberkornzahlen in der Filmschicht über den Zellkernen bestimmt. Zellkerne, die mehr als 3 Silberkörner aufwiesen, wurden als markiert gewertet. Der prozentuale Anteil markierter Zellen einer Reifungsklasse ergab den «Markierungsindex».

Nach morphologischen Kriterien wurden 8 granulopoetische Zelltypen als Kompartments unterschieden: M_1 = Myeloblast, M_2 = Promyelozyt, M_3 = halbreifer Myelozyt, M_4 = reifer Myelozyt, M_5 = Metamyelozyt, M_6 = Jugendlicher, M_7 = stabkerniger Neutrophiler, M_8 = segmentkerniger Neutrophiler. Die in Abbildung 1 angegebenen Normalwerte für die verschiedenen Kompartments sind das Ergebnis zytologischer Differenzierungen von fast 30000 granulopoetischen Zellen in 14 Knochenmarkpräparaten einer Normalperson [12].

Bestimmung der Neutrophilenspeicher und des Neutrophilenumsatzes im Blut. Etwa 500 ml Blut

Ende aus dem Infusionsschlauch Blutproben entnommen für Leukozyten-Zählungen und -Differenzierungen und zur Herstellung von Leukozytenkonzentraten. Zur Bestimmung der autotransfundierten Blutmenge wurde das gesamte Transfusionsbesteck vor und nach der Blutentnahme und nach der Reinfusion gewogen.

Unter Berücksichtigung der transfundierten Blutmenge und der Zahl markierter Neutrophiler pro µl Infusionsblut liess sich die Gesamtzahl der reinfundierten markierten Neutro-

... et al. [4] wurden für Neutrophile 3 verschiedene Speichergrossen ermittelt. Die Werte ...
ergaben sich durch M...
philen (N_S) mit deren ...
de Neutrophilenspeiche...
bezogene Neutrophilen...
ginalen Neutrophilensp... (MNS)

[1] Fa. Radiochemical Centre Amersham, spez. Akt. 5 Ci/mM
[2] Fa. Radiochemical Centre Amersham, spez. Akt. 4820 mc/mM

Tabelle I. Speichergrössen, intravasale Halbwertszeit und Umsatzrate der Neutrophilen in zwei verschiedenen Krankheitsabschnitten bei einer Patientin mit zyklischer Neutropenie. Normalbereiche nach ATHENS *et al.* [2] und ALBERTSON [1]

	Neutrophile[1]	GBNS[2]	ZNS[3]	MNS[4]	T½N[5]	NUR[6]
Remission	2400	59	16	43	5	8,2
Neutropenie	240	10	1,6	8,4	2,3	3
Normal	4500	70	31	39	6,7	6,8
	(3–7 × 10³)	(14–160)	(11–46)	(0–85)	(4–10)	(2,1–14)

[1] Neutrophile pro µl Venenblut
[2] Gesamter Blutneutrophilenspeicher (× 10⁷/kg Körpergewicht)
[3] Zirkulierender Neutrophilenspeicher (× 10⁷/kg Körpergewicht)
[4] Marginaler Neutrophilenspeicher (× 10⁷/kg Körpergewicht)
[5] Intravasale Halbwertszeit der Neutrophilen (h)
[6] Neutrophilenumsatzrate (× 10⁷/kg Körpergewicht/h)

Die intravasale Halbwertszeit der autotransfundierten Neutrophilen wurde graphisch im halblogarithmischen Koordinatensystem bestimmt (Abb. 3). Bei Kurven mit 2 Abschnitten wurde hierzu der 2. Abschnitt verwendet, d. h. es wurden lediglich die später als 2 h nach der Autotransfusion ermittelten Werte berücksichtigt.

Ein Näherungswert für die stündliche Umsatzrate der Neutrophilen (NUR) ergab sich durch Multiplikation von GBNS mit ln 2 und Division durch die intravasale Halbwertszeit der markierten Zellen.

Untersuchungen, die mit dieser Methode an der Normalperson durchgeführt wurden, ergaben ähnliche Werte wie sie von ATHENS *et al.* durch Markierung mit DF³²P und Messung der Radioaktivität der gesamten Leukozyten gefunden wurden. Zur Beurteilung der bei der Patientin gewonnenen Ergebnisse (Tab. I) wurden deshalb die von diesen Autoren angegebenen Normalbereiche [2] herangezogen.

Ergebnisse

Symptomatik und Blutzellstatus. Der klinische Untersuchungsbefund der Patientin war abgesehen von den unten beschriebenen Beschwerden, die intermittierend zusammen mit Neutropenie und Monozytose auftraten, unauffällig.

Während der Beobachtungszeit waren die Werte der Erythrozyten (um 4 Mill. pro µl Blut), der Thrombozyten (um 250000 pro µl Blut) und der Lymphozyten (um 1500 pro µl Blut) relativ konstant. Die Retikulozytenzahlen waren unverändert leicht erhöht (um 24‰ entsprechend 96000 pro µl Blut).

Der Neutrophilenspiegel im Blut schwankte periodisch zwischen leicht neutropenischen Werten und einem nahezu völligen Fehlen der Neutrophilen. Aufgrund der registrierten Neutrophilenkurve (Abb. 1) und der beobachteten Symptomatik wurde der Krankheitszyklus in folgende 4 Abschnitte unterteilt:

1. Einen Abschnitt der «Remission» mit Neutrophilenwerten zwischen 1500–2500 pro µl Blut, während dem sich die Patientin völlig gesund fühlte.

2. Im folgenden «Initialabschnitt der Neutropenie» fiel der Neutrophilenspiegel ab, die Monozytenzahl stieg an, und es traten Beschwerden auf, die mit dem Grad der Neutropenie zunahmen: Müdigkeit, Leistungsschwäche, Appetitlosigkeit, Brechreiz, Erbrechen, nicht lokalisierbare Oberbauchbeschwerden und Fieber.

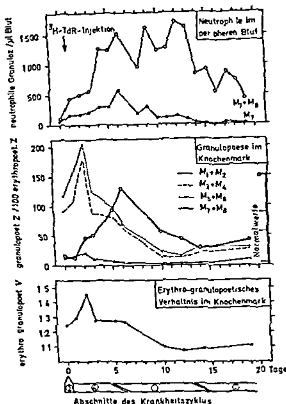


Abb 1. Neutrophilenspiegel im Blut, Granulopoese und erythro-granulopoetisches Verhältnis im Knochenmark während einer Phase einer zyklischen Neutropenie (Normalwerte nach FLIEDNER [12]) M_1 = Myeloblast, M_2 = Promyelozyt, M_3 = halbreifer Myelozyt, M_4 = reifer Myelozyt, M_5 = Metamyelozyt, M_6 = Jugendlicher, M_7 = stabkerniger neutrophiler Granulozyt, M_8 = segmentkerniger neutrophiler Granulozyt. Krankheitsabschnitte *1* Remission, *2* Initialabschnitt der Neutropenie, *3* Zeitpunkt der maximalen Neutropenie, *4* Reparation.

Ein Anhalt für das Vorliegen eines chronischen oder für das zeitweilige Auftreten eines akuten Infekts war nicht gegeben. Im Status febrilis zweier verschiedener Krankheitszyklen wurde die Serumkonzentration von Ätiochonanolon und einmal auch von 11 Keto-Pregnanolon bestimmt. Die Werte waren erhöht. In fieberfreien Abschnitten des Krankheitszyklus lagen Normalwerte vor

3 Im [illegible]
100 pro µl [illegible]
H hepunkt [illegible]
den Beschw [illegible]

4. Nach diesem etwa 1 bis 2 Tage andauernden Zustand setzte mit dem 4. Krankheitsabschnitt ein Anstieg der Neutrophilen und ein Abfall der Monozyten ein, verbunden mit einem schliesslich vollständigen Verschwinden der Krankheitssymptome (»Reparation«).

Die Phasendauer zweier kontinuierlich beobachteter Zyklen lag im Mittel bei 26 Tagen. Etwa 12 Tage entfielen auf die Remission, 9 Tage auf den Initialabschnitt der Neutropenie und 5 Tage auf den Abschnitt der Reparation.

Zytologische Befunde im Knochenmark. Aufgrund der relativ konstanten Erythrozyten- und Retikulozytenwerte im peripheren Blut wurde eine während des Krankheitsgeschehens gleichbleibende Erythropoese angenommen, zu der die Befunde der Granulopoese in Beziehung gesetzt wurden. Die derart umgerechneten Ergebnisse von Knochenmark-Differentialzählungen (1500–3000 kernhaltige Zellen pro Zeitpunkt) sind in Abbildung 1 dargestellt. Es ergaben sich Kurven, die erkennen lassen, dass synchron mit dem Ansteigen und Absinken des Neutrophilenspiegels im Blut auch bei der Granulopoese im Knochenmark quantitative Veränderungen abliefen. Die übrigen Zellsysteme des Knochenmarks verhielten sich unauffällig.

Zu Beginn der Reparation (2. Tag) erreichte die Gesamtzahl granulopoetischer Zellen das Maximum. Das erythro-granulopoetische Verhältnis betrug in diesem Abschnitt 1 : 4,5. Die Reifungsstufen M_1–M_4 waren auf das 2–3fache der Norm vermehrt, die reiferen Formen M_7 und M_8 waren im Gegensatz hierzu, ähnlich wie im peripheren Blut, stark vermindert.

Im weiteren Verlauf der Reparation konnte eine rasche Verschiebung innerhalb der granulopoetischen Zellen zugunsten der reiferen Formen beobachtet werden. Um den 4. Tag dieses Abschnittes lag ein normales Myelogramm vor. Gegen Ende der Reparation (5.–6. Tag) und während der Remission (etwa 6.–14. Tag) nahm die Zahl sämtlicher Reifungsstufen der Granulopoese zunächst rascher, dann langsamer ab, bis zu einem Minimum am Übergang der Remission in den Initialabschnitt der Neutropenie. Zu diesem Zeitpunkt lag eine hypoplastische Granulopoese vor, deren Gesamtzahl nur noch etwa $^1/_3$ der Norm erreichte.

Zellkinetik der Granulopoese während der Reparation. Etwa 1 Tag nach dem Zeitpunkt der minimalen Neutrophilenzahl im Blut wurde der Patientin ^{3}HTDR injiziert. Die Veränderungen der Markierungsindizes granulopoetischer Vorstufen während der ersten 12 Tage nach der Injektion von ^{3}HTDR sind in Abbildung 2 dargestellt. Zum Vergleich wurden in die Diagramme Befunde einer ^{3}HTDR-Untersuchung bei einem Patienten mit normaler Hämatopoese eingezeichnet [12].

Die Ergebnisse im Proliferationsspeicher der Granulopoese (Zellformen M_1–M_4) wichen nicht signifikant von den Befunden eines hämatologisch gesunden ab. Eindeutige Unterschiede ergaben sich jedoch bei den reiferen, initial nicht markierten Zellkompartments der Granulopoese (Zellformen M_5–M_8). Der Einstrom markierter Zellen aus dem Proliferationsspeicher in die Speicher M_5 und nachfolgende erfolgte wesentlich früher als normalerweise. Die ersten markierten jugendlichen Neutrophilen (M_6) wurden schon 8,5 h nach der ^{3}HTDR-Injektion beobachtet. Markierte stabkernige Neutrophile (M_7) fanden sich in den Knochenmarkpräparaten 15 h, segmentker-

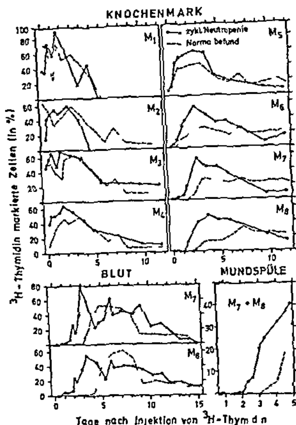


Abb. 2 Verhalten der Markierungsindizes nach intravenöser Injektion von ^{3}H Thymidin bei granulopoetischen Zellen im Knochenmark bei neutrophilen Granulozyten im peripheren Blut und im Sediment der Mundspülflüssigkeit (M_1–M_8 siehe Legende von Abb. 1)

nige Neutrophile (M_8) 38 h nach der Injektion (Normalwerte [12] $M_6 = 12$ $M_7 = 48$, $M_8 = 60$ h) Der höchste Markierungsindex wurde bei M_6 und M_7 38 h bei M_8 61 h nach der ^{3}HTDR Applikation erreicht Diese Werte waren mehr als doppelt so hoch wie die zu gleichen Zeitpunkten gemessenen Normalwerte

Im späteren Verlauf der Untersuchung (etwa nach dem 8 Tag) fanden sich keine eindeutigen Abweichungen der Markierungsindizes von der Norm mehr

Im venösen Blut konnten 24 h nach der ^{3}HTDR Injektion die ersten markierten stab- und segmentkernigen Granulozyten (M_7 und

M_8) nachgewiesen werden 43 h nach der Injektion wurden auch in den Autoradiogrammen der Präparate aus dem Sediment der Mundspülflüssigkeit markierte neutrophile Granulozyten gefunden

Die Reifungszeit der Neutrophilen, definiert als Zeitintervall zwischen dem ersten Auftreten markierter Metamyelozyten und dem Erscheinen markierter Neutrophiler im Blut, lag zum Zeitpunkt der Untersuchung bei 13 h Sie war damit gegenüber der Norm (96–120 h [12]) auf etwa $^1/_7$ verkürzt

Intravasale Halbwertszeit, Speichergrössen und Umsatzrate der Neutrophilen im Blut Während einer Remission (2400 Neutrophile pro µl Blut) und eines Initialabschnittes der Neutropenie (240 Neutrophile pro µl Blut) wurden zellkinetische Daten der Blutneutrophilen durch Autotransfusion von ^{3}HDFP markierten Neutrophilen ermittelt Zum Zeitpunkt der ersten Untersuchung während der Remission lag die intravasale Halbwertszeit der Neutrophilen bei 5 h (Abb 3, Tab I), im neutropenischen Krankheitsabschnitt dagegen betrug sie nur 2,3 h

Während der Remission lagen die Grossen der 3 verschiedenen Neutrophilenspeicher (GBNS, ZNS, MNS) in dem von ATHENS *et al* angegebenen Normalbereich [2] Zum Zeitpunkt der 2 Untersuchung war im zirkulierenden Blut nur noch $^1/_{10}$ der Neutrophilenmenge der

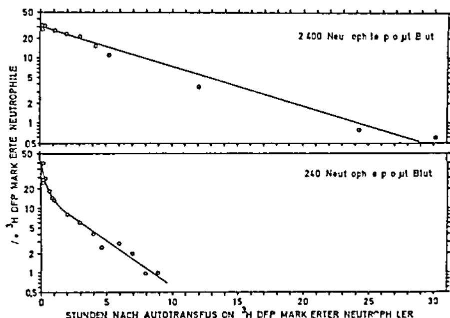


Abb 3 Markierungsindex neutrophiler Granulozyten im Blut nach Autotransfusion in vitro mit ³H DFP markierten Granulozyten in zwei Krankheitsabschnitten.

Voruntersuchung vorhanden Die Abnahme der Grösse von GBNS betrug $^5/_6$ des Wertes der Remission, bei MNS machte der Abfall $^4/_5$ aus Die Grosse der Umsatzrate dagegen fiel weniger als $^2/_3$ des Ausgangswertes ab

Diskussion

Analyse der rhythmischen Veranderungen des Neutrophilenspiegels im Blut der untersuchten Patientin

Schwankungen der Neutrophilenzahl im Blut konnen Veranderungen folgender Komponenten zugrunde liegen 1 der Zellverteilung zwischen den Neutrophilenspeichern, 2 der Neutrophilenumsatzrate, 3 der Neutrophilenproduktionsrate Der Erorterung dieser 3 Punkte werden Daten zugrunde gelegt, die durch ³HDFP markierte Neutrophile zum Zeitpunkt einer fast maximal ausgeprägten Neutropenie und wahrend einer Remission gewonnen wurden Da kein Hinweis auf eine sprunghafte Änderung der Neutrophilenkinetik vorlag (es konnten weder Infektzeichen noch Leukozytenantikorper oder Veranderungen der Milzgrosse nachgewiesen werden), kann angenommen werden, dass bei diesen Untersuchungen die wahrend des Zyklus vorkommenden Extremwerte bestimmt wurden, zwischen denen die Umsatzraten und Speichergrossen wahrend der ubrigen Abschnitte der Krankheit lagen

Zu 1 Analysiert man die ermittelten Grossen der Neutrophilenspeicher, so findet man wahrend der Neutropenie einen im Vergleich zur Remission um 90% verminderten Zellgehalt im ZNS, dem ein Abfall von nur 83% im GBNS gegenubersteht (Tab II) Das Ver-

Tabelle II Verhalten der Neutrophilenspeicher während einer Remission und einer fast maximal ausgeprägten Neutropenie

	GBNS[1]	ZNS[1]	GBNS ZNS	[ZNS][2]
A. Remission	59	16	1 0 27	16
B. Neutropenie	10	1 6	1 0 16	2,24
Abfall (A B)	83%	90%	40%[3]	86%

[1] GBNS und ZNS = Zellen × 10^7/kg Körpergewicht
[2] [ZNS] = ZNS wenn GBNS ZNS = 1 0,27
[3] Abfall von ZNS als Anteil von GBNS

hältnis von GBNS zu ZNS hatte sich also während der Neutropenie zugunsten von GBNS verschoben. Wird diese Zellverschiebung zwischen den Speichern rechnerisch eliminiert, so würde sich für ZNS ein Abfall von 86% ergeben, der ausschliesslich Folge des Zellverlusts von GBNS war. Aufgrund dieser Analyse kann gesagt werden, dass die Neutropenie fast ausschliesslich Folge einer Verkleinerung des Gesamtspeichers der Neutrophilen und nur zu 4% durch eine Änderung der Zellverteilung in den Speichern bedingt war.

Zu 2: Die Ursache des zyklisch auftretenden Neutrophilenabfalls war mit Sicherheit nicht ein erhöhter Neutrophilenuntergang, da die Neutrophilenumsatzrate während der neutropenischen Krankheitsabschnitte nicht anstieg, sondern abfiel.

Zu 3: Eine Vorstellung der Neutrophilenproduktionsrate während des Krankheitszyklus soll mit Hilfe des in Abbildung 4 dargestellten Modells der Granulopoese gewonnen werden. Es besteht aus hintereinandergeschalteten Kompartments, in denen sich die verschiedenen Zellformen der Granulopoese während der Proliferation, Reifung und Speicherung jeweils eine bestimmte Zeit aufhalten. Aus der Grosse des Zelleinstroms in das Kompartment und des Zellabstroms resultiert entweder ein Fliessgleichgewicht oder eine Änderung der Zellzahl in diesem. Vorausgesetzt, dass innerhalb des Speichers keine Zellen zugrunde gehen, gilt folgende Beziehung: Änderung der Speichergrosse = Zelleinstrom – Zellabstrom oder: Zelleinstrom = Änderung der Speichergrosse + Zellabstrom (Daten in Zellen pro Zeiteinheit). Durch die Neutrophilenumsatzrate (NUR) und die Änderung des Gesamt-Blutneutrophilenspeichers (GBNS) lässt sich mit dieser Formel zunächst die Rate des Neutrophileneinstroms ins Blut bestimmen. Diese Grosse, eingesetzt als Neutrophilenabstrom aus dem Neutrophilenspeicher des Knochenmarks, ergibt zusammen mit der Änderung der Grosse dieses Kompartments die Produktionsrate von Neutrophilen aus den Präkursoren.

Mit den zur Verfügung stehenden Daten wurde diese Analyse folgendermassen durchgeführt. Die Kurve der Neutrophilenumsatzrate während des Krankheitszyklus in Abbildung 4 ergab sich unter den oben näher ausgeführten Voraussetzungen. Die Daten von GBNS und ZNS (Tab. II) lassen es als wahrscheinlich erscheinen, dass sich die Speicher während der Krankheitsphasen annähernd parallel änderten. Zur Ermittlung der Neutrophileneinstromrate ins Blut konnte also auch die Änderung von ZNS in die Formel eingesetzt werden.

Das Verhalten von ZNS während des Zyklus ergab sich aus einer Kurve, die an den Neutrophilenzahlen im Blut orientiert war. Hierbei wurde, gestützt auf die relativ konstanten

Werte der Erythrozyten, der Retikulozyten und des Hämatokrits vorausgesetzt, dass sich das Blutvolumen während des Zyklus nicht wesentlich veränderte. Mit Hilfe dieser Kurve (Abb. 4 «Neutrophile im Blut») konnte für jeden Zeitpunkt (t) des Zyklus ein Mass für die Speichergrösse (y) bzw. für die tägliche (Δt = konst.) Änderung der Speichergrösse (Δy) abgelesen werden.

Die graphisch durchgeführte Addition von Δy zu den Werten der Neutrophilenumsatzrate in den entsprechenden Zeitpunkten ergab eine Kurve für die Einstromrate der Neutrophilen ins Blut (Abb. 4 «Neutrophilen Einstrom ins Blut»). Die Grösse der Neutrophilenproduktion ergab sich schließlich durch graphische Addition der Änderungsraten des Neutrophilenspeichers im Knochenmark zu der Kurve des Neutrophileneinstroms ins Blut (Abb. 4 «Neutrophilenbildungsrate im Knochenmark»).

Durch diese zellkinetische Analyse konnte gezeigt werden, dass die Neutrophilenproduktion rhythmisch verlief, mit derselben Phasendauer wie die zyklischen Veränderungen des Neutrophilenspiegels im Blut. Den Schwankungen der Neutrophilenproduktion folgten jeweils gleichgerichtete Veränderungen des Neutrophilenspiegels im Blut nach. *Die bei der untersuchten Patientin beobachteten zyklischen Veränderungen des Neutrophilenspiegels waren somit Folge rhythmischer Schwankungen der Neutrophilenproduktion im Knochenmark.*

Zellkinetik und funktionelles Verhalten der Granulopoese während des Krankheitszyklus

Einen Überblick über die Befunde der verschiedenen Zellkompartments der Granulopoese im Verlauf einer Krankheitsphase vermittelt Abbildung 4. (Die Kurven wurden im letzten Teil des Intervallabschnittes der Neutropenie bis zu den Werten eines maximal neutropenischen Zustandes ergänzt.) Innerhalb des Krankheitszyklus lassen sich folgende funktionell verschiedene Abschnitte der Granulopoese unterscheiden.

Reparation (Anfang). Bei minimalen Neutrophilenwerten im Blut nahmen zunächst im Proliferations- und Reifungsspeicher die Zellzahlen rasch zu. Durch eine, wie die Befunde der ³HTDR-Untersuchung zeigten (Abb. 2), ungestörte Proliferation und stark beschleunigte Reifung setzte dann rasch eine Zunahme der Neutrophilenbildungsrate mit vermehrtem Neutrophileneinstrom ins Blut ein, die entleerten Neutrophilenspeicher im Knochenmark und im Blut wurden wieder aufgefüllt. Die vorliegende Situation der Granulopoese entspricht etwa einem experimentell herbeigeführten Zustand nach Entspeicherung der Neutrophilenpools durch Leukophorese. Dieser Ein-

hältnis von GBNS zu ZNS hatte sich also während der Neutropenie zugunsten von GBNS verschoben. Wird diese Zellverschiebung zwischen den Speichern rechnerisch eliminiert, so würde sich für ZNS ein Abfall von 86% ergeben, der ausschliesslich Folge des Zellverlusts von GBNS war. Aufgrund dieser Analyse kann gesagt werden, dass die Neutropenie fast ausschliesslich Folge einer Verkleinerung des Gesamtspeichers der Neutrophilen und nur zu 4% durch eine Änderung der Zellverteilung in den Speichern bedingt war.

Zu 2: Die Ursache des zyklisch auftretenden Neutrophilenabfalls war mit Sicherheit nicht ein erhöhter Neutrophilenuntergang, da die Neutrophilenumsatzrate während der neutropenischen Krankheitsabschnitte nicht anstieg, sondern abfiel.

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Mit den zur Verfügung stehenden Daten wurde diese Analyse folgendermassen durchgeführt. Die Kurve der Neutrophilenumsatzrate während des Krankheitszyklus in Abbildung 4 ergab sich unter den oben näher ausgeführten Voraussetzungen. Die Daten von GBNS und ZNS (Tab. II) lassen es als wahrscheinlich erscheinen, dass sich die Speicher während der Krankheitsphasen annähernd parallel änderten. Zur Ermittlung der Neutrophileneinstromrate ins Blut könnte also auch die Änderung von ZNS in die Formel eingesetzt werden.

Das Verhalten von ZNS während des Zyklus ergab sich aus einer Kurve, die an den Neutrophilenzahlen im Blut orientiert war. Hierbei wurde, gestützt auf die relativ konstanten

Werte der Erythrozyten, der Retikulozyten und des [illegible] [illegible], [illegible] das Blutvolumen während des Zyklus nicht wesentlich [illegible] [illegible] [illegible] (Abb. 4 «Neutrophile im Blut» konnte für jeden Zeitpunkt [illegible] [illegible] [illegible] [illegible] die Speichergrösse (y) bzw. für die tägliche Δ [illegible] [illegible] [illegible] (Δ y) abgelesen werden.

Die graphisch durchgeführte Addition von Δ y zu den Werten der [illegible] umsatzrate in den entsprechenden Zeitpunkten ergab eine Kurve [illegible] [illegible] Neutrophilen ins Blut (Abb. 4 «Neutrophilen-Einstrom ins Blut»). Die Grösse der Neutrophilenproduktion ergab sich schliesslich durch graphische Addition der [illegible] des Neutrophilenspeichers im Knochenmark zu der Kurve des [illegible] [illegible] Blut (Abb. 4 «Neutrophilenbildungsrate im Knochenmark»).

Durch diese zellkinetische Analyse konnte [illegible] werden, dass die Neutrophilenproduktion rhythmisch verlief, mit derselben Phasendauer wie die zyklischen Veränderungen des Neutrophilenspiegels im Blut. Den Schwankungen der Neutrophilen[illegible] folgten [illegible] gleichgerichtete Veränderungen des Neutrophilenspiegels im Blut nach. *Die bei der untersuchten Patientin beobachteten zyklischen Veränderungen des Neutrophilenspiegels waren somit Folge rhythmischer Schwankungen der Neutrophilenproduktion im Knochenmark.*

Zellkinetik und funktionelles Verhalten der Granulopoese während des Krankheitszyklus

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Reparation (Anfang). Bei minimalen Neutrophilen[illegible] im Blut nahmen zunächst im Proliferations- und Reifungsspeicher die Zellzahlen rasch zu. Durch eine, wie die Befunde der [illegible]-Untersuchung zeigten (Abb. 2), ungestörte Proliferation und stark beschleunigte Reifung setzte dann rasch eine Zunahme der Neutrophilenbildungsrate mit vermehrtem Neutrophileneinstrom ins Blut ein. [illegible] geleerten Neutrophilenspeicher im Knochenmark und im Blut wurden wieder aufgefüllt. Die vorliegende Situation der Granulopoese entspricht etwa einem experimentell herbeigeführten Zustand nach Entspeicherung der Neutrophilenpools durch Leukopherese. [illegible] [illegible]

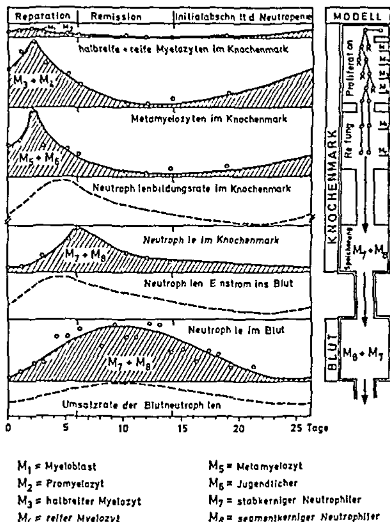


Abb 4 Halbschematische Darstellung des Verhaltens der Zahlen und der Kinetik der verschiedenen Zellformen der Granulopoese während eines Krankheitszyklus

griff wird von einer normalen Granulopoese ebenfalls mit einer Mehrproliferation und Mehrproduktion von Neutrophilen beantwortet [8].

Reparation (Mitte) Nachdem die Neutrophilenwerte im Blut der Patientin etwa 500 pro μl erreicht hatten, nahmen die Zellzahlen im Proliferationsspeicher bereits wieder ab

Reparation (Ende). Die zu Beginn der Reparation im Proliferationsspeicher vermehrt neugebildeten Zellen stromten nach der Ausreifung

in den Neutrophilenspeicher des Knochenmarks ein, der am Ende der Reparation seine maximale Zellzahl erreichte

Remission Durch den Abfall der Zellzahlen im Proliferations- und Reifungsspeicher ging die Neutrophilenbildungsrate während der Remission stetig zurück Etwa 3 Tage nach dem Maximum im Knochenmark wurden auch im Blut die höchsten Neutrophilenzahlen gezählt, die jedoch niemals Normalwerte erreichten

Die während der besprochenen Krankheitsabschnitte erhobenen Befunde lassen auf ein funktionell normales Verhalten der Granulopoese zu Beginn der Reparation schliessen Die Zunahme der Proliferation als Antwort auf die Neutropenie war jedoch «unterdosiert» (Ende der Reparation), so dass keine Normalisierung des Neutrophilenspiegels erreicht wurde (Beginn der Remission) Im Verlauf dieses Krankheitsabschnittes nahm das Missverhältnis zwischen Neutrophilenbildung und Neutrophilenspiegel im Blut weiter zu

Initialabschnitt der Neutropenie Die Neutrophilenbildungsrate und dadurch auch die Zellzahlen in den Neutrophilenspeichern fiel auf Minimalwerte ab Gleichzeitig aber nahm die Zahl der Neutrophilen-Prakursoren M_1–M_4 wieder zu Möglicherweise lag diesen Befunden eine Reifungsstörung beim Übergang der jugendlichen zu den stabkernigen Neutrophilen zugrunde Würde diese Annahme zutreffen, so könnte ein Neutrophilenanstieg bis auf Werte von 6000 pro μl, der nach einer Prednisonmedikation in dieser Situation beobachtet wurde, als eine durch das Medikament ermöglichte Ausreifung von im Knochenmark «aufgestauten» Prakursoren interpretiert werden Diese Deutung würde auch den Neutrophilenabfall erklären, der [illegible]

unter 100 pro μl Blut, sprang plötzlich die Proliferation der Granulopoese für einige Tage wieder voll an, Zeichen einer gestörten Reifung waren dann nicht mehr nachweisbar

Ein Modell zur Simulation des funktionellen Verhaltens der Granulopoese

Das granulozytäre Zellerneuerungssystem wird in Abbildung 5 als ein Modell dargestellt, in dem die einzelnen Zellkompartments mit

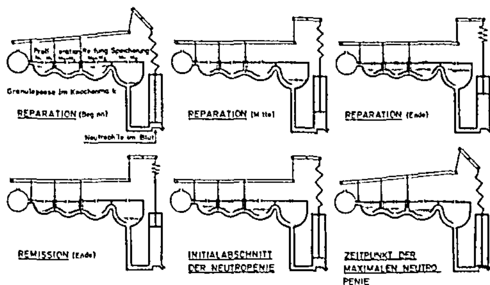


Abb. 5 Ein Modell zur Simulation der Granulopoese bei der Patientin mit zyklischer Neutropenie

Hilfe kaskadenartig hintereinander geschalteter, durch Flüssigkeitsheber verbundener Gefässe nachgeahmt werden.

Der vom Stammzellspeicher ausgehende Zellstrom muss nach Durchfliessen des Proliferations- und Reifungsspeichers zwischen den Kompartments M_6 und M_7 ein besonders hohes Niveau überwinden. Ein Regelsystem sondiert den Neutrophilenspiegel im Blut und steuert durch Ventile sowohl den Zelleinstrom in das System aus dem Stammzellspeicher als auch den Zellfluss zum Reifungsspeicher. Eine Spiralfeder erhöht die Reizschwelle der Steuerung derart, dass diese erst bei extrem niedrigen Neutrophilenzahlen anspricht und den Zellfluss für kurze Zeit freigibt. Hierauf erfolgt ein Anstieg des Neutrophilenspiegels im Blut, wodurch das Regelsystem zurück-«kippt» und erneut die Proliferationsaktivität der Granulopoese bremst.

Zellen, die während der auf einige Tage befristeten Freigabe des Zellflusses neu gebildet wurden, durchfliessen wellenartig die verschiedenen aufeinanderfolgenden Speicher. Gegen Ende der Remission haben die während der Reparation vermehrt neu gebildeten Zellen die Kompartments M_1–M_6 der Granulopoese verlassen. In diesen liegt dann ein extrem niedriger Zellspiegel vor, so dass der Zellfluss zwischen den Kompartments M_6 und M_7 abreisst. Während

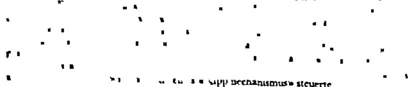
des Initialabschnittes der Neutropenie stauen sich die von den Prakursorenspeichern nachfliessenden Zellen vor dem Hindernis zwischen M_6 und M_7, bis schliesslich zu Beginn der Remission der Speicher M_6 wieder ein Niveau erreicht, das den Zelleinstrom in das Kompartment M_7 ermoglicht

Obwohl nicht beurteilt werden kann, inwieweit ein solches Modell zur Deutung der Untersuchungsergebnisse bei der Granulopoese herangezogen werden darf, scheint doch der Vorstellung einer gestorten Regulation eine gewisse Wahrscheinlichkeit zuzukommen, da sich in dieses Bild auch andere, rhythmisch auftretende Befunde einordnen lassen Ähnlich wie bei den in der Literatur dargestellten Fallen, traten auch bei dieser Patientin synchron mit den hamatologischen Veranderungen verschiedene weitere Krankheitssymptome auf, wie z B gastrointestinale Beschwerden, Fieber und depressive Verstimmung Durch eine Analyse der Ursache dieser Symptome an den einzelnen Organen selbst, von denen sie jeweils ausgehen – ahnlich wie sie hier für die Granulopoese unternommen wurde – würde sich wahrscheinlich eine grosse Zahl von Funktionsstorungen aufzeigen lassen, die, obwohl kein direkter pathogenetischer Zusammenhang erkennbar ist, rhythmisch und synchron miteinander auftreten und wieder verschwinden

Offensichtlich kann also auch zur Deutung der Pathogenese des gesamten Krankheitssyndroms eine gestorte Regulation erwogen werden Wie bei dem in Abbildung 5 dargestellten Modell der Granulopoese konnte ein vermindert ansprechbares zentrales Steuerungssystem vorliegen, das die Funktion normaler Organe nur noch in weiten Grenzen zu regeln vermag

Zusammenfassung

Bei einer Patientin mit zyklischer Neutropenie wurde mit Zellzählungs- und Differenzierungsmethoden zusammen mit verschiedenen Zellmarkierungen durch radioaktive Nuklide (^{3}HTDR, ^{3}HDFP) die Zellkinetik der Granulopoese und des Neutrophilen



mechanismus steuerte

Summary

Cellular kinetics of the granulopoiesis and the neutrophilic system were studied by total and differential cell counting methods together with labelling of various elements with radioactive nuclides (^{3}H TDR, ^{3}H DFP) in a patient with cyclical neutropenia. The periodic rise and fall of the neutrophil levels in the blood typical of the disease was shown to result from rhythmical fluctuations in the production of neutrophils in the marrow. These in turn were caused by cyclical variations in the proliferative activity of granulopoiesis which can be regarded as the expression of a disturbance of a mechanism controlling proliferation in granulopoiesis by a balance-arm system.

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L. P. Hollănder (editor) **Proceedings of the XIth Congress of the International Society of Blood Transfusion.** Bibliotheca Haematologica No 29 Part I–IV Karger, Basel/New York 1968 LXIV + 1250 p, 347 fig, 216 tab Price sFr/DM 255.–/$ 61.20/£ 25 10 0

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Acta haemat. 43 65–72 (1970)

Disturbances of Hemostasis in Acute Myeloblastic Leukemia[1]

F ROSNER, J V DOBBS, N D RITZ and S L LEE
with the technical assistance of THELMA FERNBACH M.T

Division of Hematology Department of Medicine Maimonides Medical Center and State University of New York, Downstate Medical Center, Brooklyn N Y

Patients with acute leukemia frequently have a hemorrhagic tendency Various mechanisms for this bleeding diathesis have been described including thrombocytopenia, fibrinolysis, and diminution in one or more coagulation factors

A thrombotic tendency in patients with acute leukemia also occasionally occurs and, when present, is thought to be due to a 'hypercoagulable state' in which thrombocytes [8], antihemophilic globulin [15], fibrinogen [17] and other coagulation factors are increased

The following results were observed in 49 patients with acute myeloblastic leukemia in whom coagulation studies were performed

Materials and Methods

Patient selection. Between 1962 and 1966, patients were pre-selected for coagulation screening studies. Only patients with hemorrhagic or thrombotic manifestations were studied. Since 1967 hemostatic function has been evaluated in all patients with acute leukemia at the time of admission to the hospital (prior to any antileukemic therapy) and at intervals thereafter In 23 of the 49 subjects no treatment had been administered at the time of the first coagulation evaluation With the exception of 4 persons who had acute promyelocytic leukemia all patients had typical findings of acute myeloblastic leukemia. Patients with acute lymphoblastic leukemia and chronic leukemias were excluded from this report.

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Methods Partial thromboplastin times were performed according to Proctor and Rapaport [11] Prothrombin consumption was estimated as described by Aggeler *et al.* [1] Thrombin times were determined by the method of Lewis [9] and fibrinogen levels by the ammonium sulfate technique of Colls and Roman [5] In most patients the clotability of fibrinogen was also measured [12]

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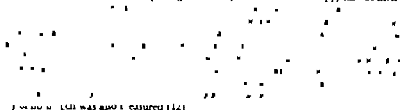
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Specific assays for factor II (prothrombin) and factor V (proaccelerin) were done by the methods of HJORT *et al.* [7] and BORCHGREVINK *et al* [4] respectively. Factors VII and X (proconvertin and Stuart factor) were assayed together and factor X alone according to DECKERT [6] Factor VIII (antihemophilic globulin) was estimated by slight modification of the one stage assay of RAPAPORT *et al* [13] and factor XIII (fibrin stabilizing factor) was determined as described by LORAND and DICKENMAN [10]

Euglobin lysis time was determined by the method of SHERRY *et al* [16] and Astrup plate lysis was measured according to ASTRUP and MULLERTZ [2] Casein proteolytic assay was estimated according to RITZ and RUBIN [14].

Results

A. Tests of Platelet Function and Vascular Integrity

Of 49 patients platelet counts were below 50,000/mm³ in 24 patients at the time of first evaluation; 5 patients had counts between 40,000 and 50,000, 5 between 30,000 and 40,000, 7 between 20,000 and 30,000, 4 between 10,000 and 20,000 and 3 below 10,000/mm³. Ten subjects had platelet counts between 50,000 and 100,000, and in 15 patients the initial platelet count was greater than 100,000/mm³ but less than 150,000/mm³.

Clot retraction was poor in 19, fair in 9 and good in 21 patients in spite of some degree of thrombocytopenia in 43 of 49 patients.

Qualitative tests of platelet function were performed in 5 individuals. These tests included platelet adhesiveness, platelet aggregation with added adenosine diphosphate, and a modified thromboplastin generation test comparing normal platelets with the patient's platelets. Qualitative platelet abnormalities were detected in only one patient (fig. 1).

The bleeding time was normal in 15 patients in all of whom platelet counts were abnormal (25,000/mm³ to 140,000/mm³). In the 18 patients with prolonged bleeding times, seven values were greater than 10 min. The prolonged bleeding times were associated with varied platelet counts ranging from 12,000 mm³ to 150,000/mm³ with four of the counts being 150,000/mm³.

B. Non-Specific Tests of Coagulation (Screening Tests)

Coagulation time. None of the 49 patients studied had a shortened coagulation time. In only 6 patients was the coagulation time abnormally prolonged.

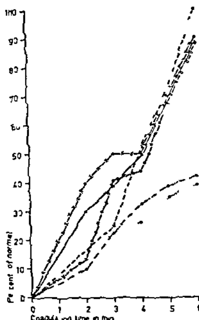


Fig 1 Thromboplastin generation test comparing normal platelets with the platelets of one patient. The patient's platelets functioned half as well as the normal platelets. — Normal plasma + normal serum + ceph 1 60 ×—× patient plasma + patient serum + ceph. 1 60 - - - patient plasma + patient serum + patient platelets —— patient plasma + patient serum + normal platelets normal plasma + normal serum + patient platelets O—O normal plasma + normal serum + normal platelets.

Silicone tube coagulation time In 3 of 38 patients the values were greater than 30 min However, in 15 patients the silicone tube coagulation times were less than 15 min Five of these patients also had elevated fibrinogen levels

Prothrombin (Quick) time This test was performed 68 times in 48 patients Forty-four determinations showed abnormally long times with 28 values between 13 and 15 s and 16 values greater than 15 s In 1 patient with a prothrombin time of 16 s, all coagulation factor assays were normal In all of the 43 remaining patients with high prothrombin times, depression of specific clotting factors were present, usually factors *II*, *V*, *VIII*, and X in various combinations or together with factor *I* These abnormalities occurred in half of the patients before therapy was initiated and in half after therapy

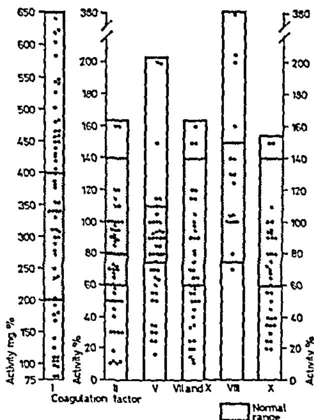


Fig. 2 Specific assays of coagulation factors in 49 patients with acute myeloblastic leukemia.

Partial thromboplastin time (PTT) In 3 patients partial thromboplastin time was shortened (less than 30 s) and in 2 of these patients fibrinogen levels were also elevated and silicone clotting times shortened In 5 other patients partial thromboplastin times were prolonged (greater than 60 s)

Prothrombin consumption test (44 patients) In all but 3 patients, platelet counts were less than 150,000/mm³ In 24 patients, less than 80% of the prothrombin was consumed In the other 20 patients normal results were observed In 7 of these patients platelet counts were below 150,000/mm³

C Specific Assays of Coagulation Factors

Fibrinogen (factor I) was determined in 45 patients Abnormally high values were observed on 24 occasions and abnormally low values were detected 13 times Four patients had fibrinogen levels greater than 600 mg/100 ml (fig 2)

Prothrombin (factor II) Eleven patients had values below 50% and 3 patients had elevated levels of prothrombin (greater than 150%) In 2 of the latter 3 patients, fibrinogen was also elevated (431 and 605 mg%) In the third patient, the fibrinogen level was at the upper limit of normal (388 mg%) but factor V was also elevated (200%) In all the patients in whom levels of prothrombin were depressed, there was also depression of factor V or one or more of the vitamin K dependent factors (factors VII, IX, X) The prothrombin time was also prolonged in all but one case

Proaccelerin (factor V) was assayed in 47 patients, it was normal in 31, decreased (below 65%) in 14, and increased (over 120%) in 2 cases In 1 of the latter 2 cases, fibrinogen, prothrombin, and anti- [illegible]

39 patients Decreased values (below 50%) were found in 10 patients A single patient had a level of 160%, this subject also had increased fibrinogen and prothrombin levels

Antihemophilic globulin (factor VIII) (17 patients) was normal in 13 patients Elevated values were found in 4 patients, in two of whom fibrinogen levels were also increased

Stuart factor (factor X) (32 patients) was normal in 23 patients In the other 9, low values (below 60%) were found Low Stuart factor levels always correlated with decreased values of the other vitamin K dependent factors and prolongation of the prothrombin time

Thrombin time (30 patients) The thrombin time was less than 10 s in 11, between 10 and 15 s in 15 and greater than 15 s in 4 patients

Fibrin stabilizing factor (factor XIII) Normal values were found in 10 of 15 patients and increased values in the other 5 patients There seemed to be no correlation between levels of factor XIII and other coagulation factors

D Tests of Fibrinolytic Activity

Whole clot lysis was observed in only 1 of the 49 patients Euglobulin lysis time was normal in all 12 patients tested The casein assay was abnormal in only 1 of these 12 patients and this patient also had increased fibrinolysis observed on the Astrup plate In 17 other patients, no increased fibrinolytic activity was present on the Astrup plates

Discussion

In our observations on 49 patients with acute myeloblastic leukemia no uniform coagulation disturbance was found although several patterns of hemostatic abnormality were found

Thrombocytopenia was a predominant finding (43 of 49 cases) but a bleeding tendency or overt purpura was often absent The bleeding tendency did not necessarily follow the degree of thrombocytopenia Three of 6 patients with normal platelet counts had prolonged bleeding times and a hemorrhagic tendency whereas 11 of the 43 patients with thrombocytopenia had normal bleeding times and no bleeding diathesis None of the other tests performed including clot retraction or prothrombin consumption was more useful than the platelet count in estimating a bleeding tendency Bleeding due to thrombocytopenia in acute leukemia is diminishing in frequency due to the availability in most large medical centers of platelet transfusions

Depression of clotting factors was the next most common abnormality encountered and was present in 9 patients with factors II, V, VII, X depressed In 4 other patients, concentrations of 2 or 3 of these factors were decreased In all patients, the decrease of these clotting factors was not directly related to therapy since these findings of coagulation disturbances were present in half of the patients before antileukemic drugs had been administered

Qualitative platelet abnormalities causing hemoptysis and purpura *in the presence of normal numbers of platelets were found in one of* our patients (fig 1) (However, only in 5 patients were platelet function studies made)

Hemorrhagic tendency in association with a reduced blood content of fibrinogen may be due to *in vivo* activation of fibrinolysis or to *in vivo* deposition of fibrin (or to a combination of both mechanisms) Earlier studies of patients with acute leukemia by other authors purport to show different mechanisms at play under apparently identical clinical circumstances Some authors relate the bleeding tendency in acute promyelocytic leukemia to consumption, while others emphasize the role of fibrinolysis Both consumption coagulopathy and fibrinolysis *have also been named as cause for occasional instances of hypofibrinogenemia* in association with non promyelocytic leukemias

In the presented series, 1 of 4 patients with promyelocytic leukemia showed elements of this syndrome and in 1 of these fibrinolysis was

prominent Ten other patients with other morphologic pictures also had some degree of hypofibrinogenemia. In this series as in most reported instances fibrinolysis appears to be an infrequent primary mechanism for bleeding, its pathogenetic role even when it occurs is not clear

Intravital fibrin deposition may present itself as hemorrhagic tendency, or with thrombotic phenomena Elevated levels of platelets or of plasma coagulation factors may be found when a thrombotic tendency is apparent Increased levels of antihemophilic globulin in acute leukemia occur [15] Markedly elevated levels of multiple clotting factors and diffuse intravascular coagulation at *post mortem* have occasionally been found [3]

One of the patients in the present study did show clinical manifestations of the 'hypercoaguable state' in the form of thrombophlebitis and possibly cerebral thrombosis This patient not only had elevated fibrinogen and factor VIII levels but also marked elevations of prothrombin and factor V Abnormally high fibrinogen levels were found in 21 of the 45 patients where this test was performed. Furthermore, 4 of 17 patients tested had significantly elevated factor VIII levels These findings help support the concept that hypercoagulability in patients with acute myeloblastic leukemia may be frequently accompany the disease This concept is predicated on the assumption that an elevation of the plasma clotting factor in the non-activated form is evidence of hypercoagulability All the measurements reported in the present study are of the non-activated species

Summary

Disturbances of hemostatic function in patients with acute leukemia take four forms [illegible]

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Fine Structural Features of Lymphocyte Transformation Induced by Mercuric Salts

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Mercuric salts, i.e. mercuric chloride, mercuric cyanide, mercuric acetate, mercuric nitrate and some organic mercurial compounds such as merbromin and oxymercurisalicylic acid induce non specific transformation and mitosis in human peripheral lymphocytes *in vitro* [10, 11, 12]. The transformed cells range from 10–25 μm in diameter and have basophilic cytoplasm. The nucleus contains up to 3 nucleoli and the chromatin has a fine reticular pattern (fig. 1 and 2). The mercury stimulated lymphocytes show enhanced RNA and DNA synthesis as demonstrated by autoradiographic technique (fig. 3) [11]. Mercuric chloride has been shown to cause non specific transformation of lymphoid cells from lymph nodes of guinea pig and rat, and from rabbit spleen, too [8]. Previous studies by light microscopy demonstrated that transformation induced by mercurial compounds is morphologically analogous to transformation mediated by phytohemagglutinin (PHA) [10, 11]. The degree of transformation induced by mercurial compounds is lower than in PHA stimulation.

Mercury stimulated lymphocytes have not yet been examined by means of electronmicroscopy. It is the purpose of the present report to describe and compare the ultrastructure of mercury stimulated and PHA stimulated lymphocytes.

Materials and Methods

Heparinized venous blood (40 units/ml) was obtained from 5 normal adult males and 2 normal adult females. Following spontaneous sedimentation of red cells, the leucocyte-rich plasma was drawn off and passed over a column of fine-mesh perlon wool (Farbwerke

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Acta haemat 43 73-9 (1970)

Fine Structural Features of Lymphocyte Transformation Induced by Mercuric Salts

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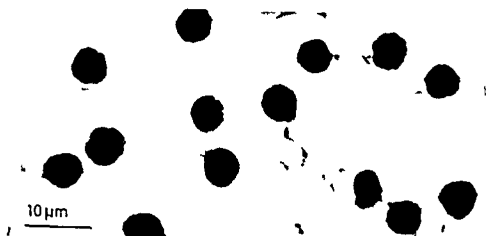
Department of Dermatology (Director Prof J. KIMMIG) University of Hamburg, Hamburg

Mercuric salts, i e mercuric chloride, mercuric cyanide, mercuric acetate, mercuric nitrate and some organic mercurial compounds such as merbromin and oxymercurisalicylic acid induce non specific transformation and mitosis in human peripheral lymphocytes *in vitro* [10, 11, 12] The transformed cells range from 10–25 μm in diameter and have basophilic cytoplasm The nucleus contains up to 3 nucleoli and the chromatin has a fine reticular pattern (fig 1 and 2) The mercury stimulated lymphocytes show enhanced RNA and DNA-synthesis as demonstrated by autoradiographic technique (fig 3) [11] Mercuric chloride has been shown to cause non specific transformation of lymphoid cells from lymph nodes of guinea pig and rat, and from rabbit spleen, too [8] Previous studies by light microscopy demonstrated that transformation induced by mercurial compounds is morphologically analogous to transformation mediated by phytohemagglutinin (PHA) [10, 11] The degree of transformation induced by mercurial compounds is lower than in PHA stimulation

Mercury stimulated lymphocytes have not yet been examined by means of electronmicroscopy It is the purpose of the present report to describe and compare the ultrastructure of mercury stimulated and PHA stimulated lymphocytes

Materials and Methods

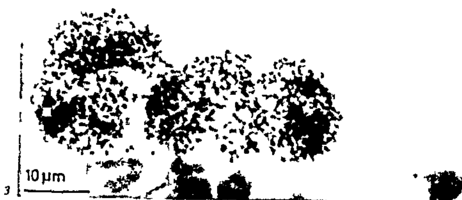
Heparinized venous blood (10 units ml) was obtained from 5 normal adult males and 2 normal adult females Following spontaneous sedimentation of red cells the leucocyte-rich plasma was drawn off and passed over a column of fine mesh perlon wool (Farbwerke



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Hoechst Germany) which resulted in a cell suspension containing 80–95% lymphocytes Aliquots of 4 ml containing 25% autologous plasma and 75% tissue culture medium TC/199 (Difco Lab Detroit USA) were placed in capped plastic culture tubes (Falcon Plastics, Los Angeles USA) to give a final cell density of 1 1.5 × 10⁶ leucocytes per ml The cultures were incubated at 37°C with mercuric nitrate or mercuric chloride (final concentration 3 5 × 10⁻⁴ M) or 2 µg ml phytohemagglutinin P (Difco Lab Detroit USA) and normal saline solution respectively as control cultures Cells were harvested 96 h later Following centrifugation at 4°C (80 g/15 min) the supernatant was discarded, the cell button suspended in Palade's osmium tetroxide fixative for 1 h at 4°C, and the resulting cell suspension [illegible]

washed with Veronal acetate buffer (pH 7 2) for 20 min dehydrated and embedded in [illegible]

Results

The most prominent fine structural features of transformed lymphocytes include the enlargement of the cells, their nuclei and nucleoli, the proportional shift of heterochromatin to euchromatin, the increase of ribosomal particles, and pseudopodium formation [6, 7, 13] These changes can be observed regardless of the mode of stimulation (fig 4 and 6) The main difference between mercury- and PHA stimulated cells was a more prominent aggregation of free ribosomes into polyribosome complexes after incubation with mercuric salts (fig 4 and 5) Other ultrastructural changes which have been described after PHA stimulation could also be observed after incubation with mercuric salts Number and distribution of organelles in transformed cells varied greatly The cytoplasm sometimes contained hardly any organelles besides polyribosomes (fig 5) Nevertheless, more mitochondria usually could be observed in transformed than in not transformed cells

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Fig 1 Lymphocytes cultured in mercury free medium (control) (× 1,500)

Fig 2 Transformation and mitosis of lymphocytes induced by mercuric chloride (3.5 × 10⁻⁴ M (× 1 500)

Fig 3 Autoradiographs of mercury-stimulated lymphocytes Incorporation of ³H-uridine showing RNA-synthesis (× 1 500



Fig 4 Cell transformed by mercuric nitrate The abundant cytoplasm contains many polyribosomes and only a few other organelles Pseudopodium formation is present (P) Nucleoli are prominent (No) The chromatin appears dispersed (× 12,000)

Fig 5 Parts of two cells transformed by mercuric nitrate The cell below displays many

Prominent Golgi complexes and a great deal of micropinocytotic vesicles were very often present The transformed cells sometimes exhibited multivesicular bodies and several bundles of perinuclear microfibrillar structures Most had a few randomly distributed strands of rough surfaced endoplasmic reticulum as well as variable fat laden vacuoles A variety of electron-dense, membrane bound cytoplasmic organelles, morphologically fulfilling the criteria for lysosomes were observed in transformed lymphocytes Definite phagocytic activity could never be found in our material

All these features of fine structure except the accumulation of polyribosome complexes, were present also in non transformed cells of cultures incubated with mercuric salts as well as in control cultures (fig 7) However, in transformed cells an increased number and a variable distribution of cell organelles could be observed There remained cells transformed by PHA or mercuric salts which strikingly resembled monocytes Besides, there were many intermediate forms, and cells which defied morphological classification

Discussion

The evaluation of the morphological changes induced by either mercuric salts or PHA is difficult for culturing alone without any transforming substances can yield a limited proliferation of lymphocytes ('background stimulation') The morphological differences between transformed and non transformed cells were quantitative rather than qualitative The vast increase of the polyribosomes was of interest, because it indicated enhanced RNA and protein synthesis confirmed by autoradiographic studies on mercury stimulated lympho-

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polyribosomes but no increase in the number of other organelles The cytoplasm of the cell above contains numerous different organelles *M* mitochondrion *MB* multivesicular bodies? (×3 000

Fig 6. Cell transformed by phytohemagglutinin Aggregation of ribosomes is not as conspicuous as after stimulation by mercuric salts (arrows Otherwise the morphological features strikingly resemble the ultrastructure of mercury transformed cells. *ER* some strands of rough-surfaced endoplasmic reticulum *No* nucleolus, *M* mitochondria (×12 000)

Fig 7 Lymphocytes from a control culture without any stimulating agent, showing only a few evenly scattered ribosomes. These cells are smaller than stimulated cells. The cell below has ample cytoplasm and a prominent Golgi complex (C) *E* erythrocyte *F* cytoplasmic fibrils, *No* nucleolus (×12 000

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Identity of Erythroblast Precursors in Bone Marrow[1]

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There is no agreement as yet on the identity of the undifferentiated erythroid precursor cell[2], although cells morphologically of the lymphocytic series have been attributed this function on the basis of indirect evidence [1–7] In an attempt to gain further information about the identity of precursors to erythroblasts, the effects of erythropoietic stimulation were studied in bone marrow in which erythropoiesis had previously been depressed.

In the majority of reports the assessment of the response of erythropoietically depressed marrow to restimulation is usually confined to observations on the blood or, in the marrow, to changes in the erythroid population itself These experiments as well as our earlier studies suggested that on restimulation of erythropoiesis an influx of cells took place into the erythroid compartment The aim of the present investigation was primarily to identify these cells by correlating quantitative changes in non-erythroid cell populations with the erythroid response

Materials and Methods

Polycythemia and erythropoietically suppressed bone marrow was produced in guinea pigs using the procedure described previously [8] Restimulation of erythropoiesis was effected by bleeding on the 9th day of rebound while unbled polycythemic guinea pigs served as controls Changes in nucleated bone marrow cells were studied in the same series of stimulated and control guinea pigs as for which the blood and marrow reticulocyte data have already been presented [8]

[1] Supported in part by NIH grant No AM/CA13145 to C Rosse and in part by research funds from the Department of Anatomy University of Bristol England

[2] The term 'erythroid precursor cell' is used to designate cells which are morphologically not identifiable as erythroblasts but have the potential to differentiate into erythroblasts

The marrow was examined by a quantitative technique previously described [9] and critically assessed [10, 11] Differential counts of marrow cells were made by identifying 1 000 nucleated cells in each animal on smears stained with MacNeal s tetrachrome stain. Morphological criteria for cell identification were as previously described and illustrated [12, 13] In addition, for each bled animal the mitotic index of erythroblasts was determined by recording the incidence of mitosis among 1 000 erythroid cells which were classified into early (proerythroblasts plus basophilic erythroblasts) and late (polychromatic plus orthochromatic) erythroblasts

The white cell count was determined on mixed venous and arterial blood obtained from the inferior vena cava and aorta at the time of exsanguination Differential counts were also performed classifying 500 leukocytes in each animal. Red cell parameters and changes in the spleen have been reported separately [8] The weight of the suprarenal glands was recorded in the stimulated animals

The results are expressed as mean values ± standard deviations The data were compared using Student s 't' test and changes were regarded significant if $p < 0\ 05$ and highly significant if $p < 0\ 001$

Results

The total marrow cellularity showed a continuous fall in the unstimulated animals during rebound ($1\ 84 \pm 0\ 02 \times 10^6 \rightarrow 1\ 40 \pm 0\ 2 \times 10^6/mm^3$ marrow), whereas in bled animals the change in cellularity was not significant ($1\ 84 \pm 0\ 02 \times 10^6 \rightarrow 1\ 67 \pm 0\ 40 \times 10^6/mm^3$) Marked quantitative changes occurred in both groups, however, within the erythroid and lymphocytic cellular compartments

Erythroid cells (fig 1) The markedly reduced number of erythroblasts [illegible]

[illegible] first erythroid increase on day 1½ was not statistically significant but from the 3rd to the 5th days both early and late erythroblasts were elevated to a level highly significant above that in the controls The number of proerythroblasts rose from $11,000 \pm 6,000/mm^3$ on day 2½ to $42,000 \pm 5,000/mm^3$ on day 3 after bleeding ($t = 5\ 50$, $p < 0\ 001$) The increase in late erythroblasts from the 1st to the 5th day was more than tenfold.

The erythroid mitotic index (fig 2) In the normal guinea pig the mitotic index of early erythroblasts was found to be $4\ 25 \pm 0\ 78\%$ and of the polychromatic erythroblasts $2\ 75 \pm 0\ 93\%$ [14] On the 9th day of rebound the mitotic index was greatly reduced in both the [illegible] ($1\ 5 \pm$ [illegible]

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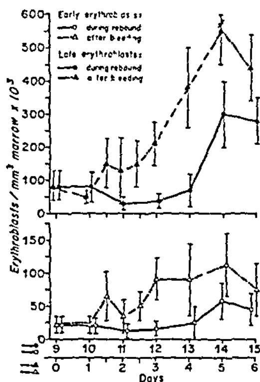


Fig. 1. Number of early (proerythroblasts + basophilic erythroblasts) and late (polychromatic + orthochromatic) erythroblasts in the bone marrow of polycythemic guinea pigs after bleeding and in the unstimulated control (rebound) Mean values ± standard deviation.

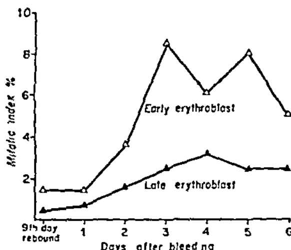


Fig. 2. Mitotic index of erythroblasts in the bone marrow of polycythemic guinea pigs following restimulation of erythropoiesis Note increase in late erythroblast mitotic index is delayed in comparison to early erythroblast Mean values

$8.4 \pm 2.2\%$ was reached ('t for 9th day rebound to 3rd day = 15.6, $p < 0.001$) and a return toward normal values occurred on the 6th day. The first significant increase in late erythroblast mitotic index was recorded only on the 4th day ($3.2 \pm 2.3\%$, $t = 3.4$, $p < 0.01$).

Lymphoid cells (fig. 3). On the 9th day of rebound the numbers of bone marrow small lymphocytes and transitional cells [12] were significantly above the normal. Small (pachychromatic) lymphocytes showed a highly significant decrease ($t = 4.81$, $p < 0.001$) in both the stimulated and unstimulated animals. In contrast, transitional cells followed trends significantly different in the bled and unbled groups. Whereas in the unstimulated controls, transitional cells declined steadily during the 6-day period after bleeding an abrupt decrease occurred approximately to the same level in the span of 24 h, and continued to day $2\frac{1}{2}$ ($t = 3.00$, $0.01 > p > 0.001$). The increase on

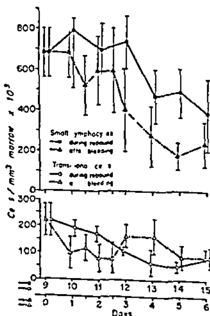


Fig. 3. Number of pachychromatic small lymphocytes and transitional cells in the bone marrow of polycythemic guinea pigs after bleeding and in the unstimulated control (rebound). Mean values ± standard deviation.

the 3rd and 4th days elevated their numbers to a level significantly above the controls ($t = 2.43$, $p < 0.02$ and $t = 2.76$, $p < 0.02$ respectively).

Other cells. Myeloid cells showed a significant increase from the 11th to the 13th day of rebound in unbled animals, while the only significant myeloid change in the stimulated group was a decrease in myeloblasts during the first 24 h after the hemorrhage ($t = 2.17$, $p < 0.05$). Myeloblasts returned to the normal level on the following day. Examination of smears and 2 μ thick sections stained with Dominici's stain did not show detectable quantitative changes in monocytes, reticulum cells, macrophages or megakaryocytes. Damaged cells in smears remained around the values found in normal marrow [13].

White cell count and weight changes in suprarenal glands. In bled animals, in spite of the hemodilution, there was a transient but significant neutrophilia on the first day while in the controls no change was noted. On this day in the bled group there was also a fourfold increase in circulating eosinophils ($t = 2.24$, $p < 0.02$). An elevated lymphocyte count was maintained in both groups throughout the experiment.

The suprarenal glands showed a significant increase in weight on the 1st day after bleeding (120 ± 33 mg $\rightarrow$ 175 ± 20 mg). The weight of these glands did not return to the initial level during the observation period.

Discussion

Stress effects of experimental procedure. Although the animals were in apparently good health, a significant increase in the weight of the suprarenal glands 24 h after the hemorrhage suggests that they may have been under considerable stress. The peripheral eosinophilia and persistent lymphocytosis do not support this contention nor can the fall of lymphocytes in the stimulated marrow be attributed entirely to stress, since a similar decrease occurred in unbled animals. In spite of the known depressant effect of corticosteroids on marrow lymphocytes [15], the weight increase in the suprarenal glands seems more likely to be due to activity directed toward stabilization of the disturbed fluid balance.

The quantitative erythroid increase. The total erythroid output by the marrow may be determined from the knowledge of (a) the bone marrow [16] and blood volume [17], (b) the daily physiological loss in red cells [18], (c) the increase in the red cell count [8], (d) the number of marrow reticulocytes [8], and (e) the number of erythro-

blasts During the first 3 days following bleeding, 7×10^8 cells were produced by the marrow, splenic red cell output at this time being negligible If these cells were all derived from the 6×10^7 mitotable erythroblasts present in the marrow at the time of bleeding, 8 to 9 divisions would have been necessary in the maturation pathway Since no increase was detected in any erythroid parameter in blood or marrow or in mitotic index up to 36 h after the stimulus, these divisions would have had to take place during the following 36 h, giving an over all turnover time of 4 to 5 h Even if the shortest reported erythroblast turnover times are accepted [19] and even if no allowance is made for 'skipped division' shown to occur under conditions similar to the present study [20] the recorded increase in the erythron can only be accounted for by a substantial influx of precursor cells into the erythron within a relatively short period of time This erythroid response is different quantitatively and in its timing from the erythroid recovery obtained in experiments using marrow shielding or transfusion for investigation of hemopoietic stem cells [21, 22]

Increase in the erythroid mitotic index While it is generally accepted that erythropoietic stimulation initiates the differentiation of increased numbers of erythroid precursor cells, the effect on the proliferation of erythroblasts is controversial Reports of the failure of anemic serum or erythropoietin to alter the rate of proliferation [23, 24] or DNA synthesis [25] in erythroblasts conflict with those where such an increase was obtained [2, 26–31] To exclude errors due to a 'shift to the left', in the present study, the mitotic index of early and late erythroblasts was recorded separately

Although the stimulus produced a measurable effect on the marrow 24 h after bleeding [8], the proliferation of erythroblasts present in the marrow at that time was apparently not affected The trends shown in figure 2 seem to represent a stream of cells entering the erythroid compartment during the period of stimulation with a significantly increased rate of proliferation A reported increase in ^{3}H thymidine incorporation by morphologically unidentified erythroid precursors soon after bleeding [32] supports this thesis It appears, therefore, that the erythropoietic stimulus had a dual effect on the precursor cell (a) it initiated its differentiation, and (b) it increased and determined its rate of proliferation

The identity of the precursor cells It is reasonable to assume that 3–4 divisions occurred during the initial 36 hours of the erythroid increase,

in which case it may be calculated that 160,000 to 80,000 cells/mm³ of marrow were required to commence erythroid differentiation during the preceding period. Changes in cell numbers of this magnitude are readily detected by the quantitative method employed.

Although the transient fall in myeloblasts following the stimulus may be related to the demands placed on the erythroid precursor cell [3], in quantitative terms this demand could have only been met by transitional cells. The total decrease in their numbers amounted to 130,000 cells/mm³ of marrow during the period preceding the first significant increase in early erythroblasts. Subsequent changes resembled the calculated response of a precursor cell to increased demands on the compartment [33]. In spite of the lack of direct evidence, the possibility of morphological transformation deduced from cytokinetic studies [5, 7], *the quantitative and chronological relationship between transitional* cells and erythroblasts, as well as the complete morphological spectrum linking the transitional cell to the proerythroblast, point to the transitional cell as the erythroid precursor or erythropoietin-sensitive cell. In normal marrow [34] and during rebound [35], transitional cells represent the major source of marrow small lymphocytes. The steady fall in their number over a period of 3 days during the late stages of rebound and following stimulation may be regarded (at present only speculatively) as a reflection of diminished lymphocyte production, since their average transit time through the marrow has been shown to be 3 days [34]. The relationship of lymphocyte precursors to erythroblast precursors, however, remains to be investigated.

Acknowledgement. The valuable technical assistance of Mr. M. FISHER is acknowledged.

Summary

Suppressed erythropoiesis was restimulated in polycythemic guinea pigs by massive bleeding. Unless unusually short cell cycle times were postulated, a large influx of cells was required into the erythron to account for the increase occurring by the 3rd day. Changes in the erythroid mitotic index indicated that erythroblasts which started maturation from the precursor cell stage had an elevated mitotic index, but the proliferation of erythroblasts present in the marrow at the time of the stimulus was not affected. Small lymphocytes showed a significance decrease in both the bled and control animals. Transitional cells followed different trends in the bled and control groups. The timing and quantitative extent of changes in these cells indicated that the cells entering on erythroid differentiation under the effect of the stimulus were most probably derived from the transitional cell compartment since no other cell could meet the requirements imposed on the erythroid precursor cell in the experiment.

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Sickle Cell Disease in Saudi Arabs

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Previous publications [9, 12] on the distribution of the sickle cell trait in Saudi Arabia have disclosed a unique geographical and ethnic localization of this hemoglobinopathy among the Shiite population of the oasis settlements along the Persian Gulf The gene frequency approaches 0 20 in some of the remote villages of the Qatif oasis, and the Shiite Saudis are believed to exhibit the highest frequency of the sickle cell trait of any population in the Middle East It is therefore not surprising that sickle cell disease (SCD) has become a major health problem of increasing importance because of the virtual disappearance of so many infectious, parasitic, and nutritional disorders which previously affected this population The economic development of the area, and advances in public health, have in greater part been derived from the operations of oil companies along the Western perimeter of the Persian Gulf These companies maintain and operate hospitals and clinics which provide medical services to large segments of the indigenous population They promote health education programs, and accommodate the health ministries of host countries with consultative support on matters of mutual interest in the field of public health It is in this setting that the problem of SCD in Saudi Arabia has evolved The reported study was carried out under the auspices of the Arabian American Oil Company (ARAMCO), and the patients encountered in clinics and hospitals provided by this company represent experience gained over a 10-year period among a group of adolescent and adult subjects with SCD

Prior to 1958, not a single case of SCD had been diagnosed in ARAMCO medical facilities, nor for that matter among Saudi Arabs

In the 10-year period, 1959–1968, the diagnosis had been verified in more than 150 Saudi children and adults. That the diagnosis was frequently overlooked in anemic subjects prior to 1958 is abundantly evident in reviewing earlier medical records. One factor which served to obscure the presence of SCD was holoendemic *falciparum* and *vivax* malaria in the oasis population [5]. The patient with anemia and splenomegaly was generally regarded as a case of malaria, even when parasites could not be demonstrated in blood films. Patients with jaundice and abdominal pain were often diagnosed as cases of viral hepatitis. An associated anemia usually failed to alert the clinician to consider the possibility of a hemolytic disorder. Finally, it was not generally appreciated that the sickle cell trait was known to occur at a significant frequency in certain Caucasian populations.

The purpose of this communication is to summarize the data which have been accumulated over a 10-year period on a group of adolescent and adult Saudi patients with SCD who have been under surveillance in ARAMCO medical facilities, to emphasize certain clinical features which distinguish this group from other reported series with more severe disease, and to consider certain genetic traits within the Saudi oasis population which may modify the expression of SCD.

Methods

Hemoglobin concentrations were determined by spectroscopic measurement of cyanmethemoglobin preparations. Red cell counts were done in duplicate by a standard counting chamber technique. Hematocrits were measured in capillary tubes by a micro-method. Reticulocyte counts were performed on wet preparations using Brecker's methylene blue stain. The range of normal for each of the above tests among Saudis is comparable with those of Caucasian Americans residing in the same area. *In vitro* sickling was determined by the metabisulphite method of DALAND and CASTLE [6]. The alkali resistant hemoglobin was measured by the method of SINGER *et al.* [20]. In normal Saudi adults the alkali resistant hemoglobin fraction was found to be less than 3.0%. Other hemoglobin components were estimated by two methods: 1) Electrophoresis on Whatmann No. 3 MM filter paper strips in a Durrum cell, with a 0.025 M barbital buffer at a pH of 8.6. Hemoglobin bands were stained with bromphenol blue-acetic acid solution. 2) Electrophoresis on Oxoid or Sepraphore III cellulose acetate strips in a Gelman horizontal cell, with 0.1 M tris-EDTA borate buffer at a pH of 8.6. Hemoglobin bands were stained with Ponceau S-acetic acid mixture. Hemoglobin components were quantitated either by elution from paper strips and spectroscopic measurement, or by densitometry using the Spinco (model RA) analytrol. With the cellulose acetate membrane separation, it was possible to estimate the percentage of A_2 hemoglobin. Only values above 4.5% were regarded as significantly elevated. Erythrocyte osmotic resistance was determined by visual estimate of the degree of hemolysis in step-wise dilutions of saline between 0.50–0.00%. For normal subjects the range was 0.43–0.34%

Table I Age and sex distribution among 65 Saudi Arabs with sickle cell disease

Age, Years	Male	Female
11-20	17	3
21-30	15	12
31-40	15	–
41-50	2	–
50	1	–
Total	50	15

NaCl from beginning to complete hemolysis In a number of instances patients were studied with various tests of hepatic function, X ray examinations of the chest, skeleton, abdomen and kidneys and electrocardiography, depending upon clinical indications.

Attempts at family investigations were often fruitless with adult patients Parents and or siblings were generally not available locally, or resided at such a distance from the laboratory as to make contact difficult Saudi employees, on occasion, fraudulantly claimed dependents in order to procure medical services which were otherwise not available in the area Polygamous marriages were fairly common, which further obscured family relationships Finally adult Saudi males were generally reluctant to submit to venipuncture in the absence of significant illness fearing impotence to be a direct consequence of unrequited blood loss

The diagnosis of SCD was based on a positive sickling preparation and the demonstration of a slow moving band of hemoglobin comprising greater than 50% of the total hemoglobin components as estimated by elution or densitometry, and corresponding in position to a reference standard of hemoglobin S (Hyland Laboratories)

Subjects

All patients were representatives of the oasis Shute population from the Eastern province of Saudi Arabia Most of the adult males were active and gainfully employed 30 of these subjects were ARAMCO employees Twenty five subjects were dependents of ARAMCO employees, and 10 had no company affiliations but had been referred by local physicians or government agencies to the ARAMCO medical department A total of 65 unrelated subjects have been included in this study, and the age and sex distribution is summarized in table 1

Clinical Features

Patients presented as cases of unexplained anemia, recurrent bone and/or joint pain, occasionally with abdominal or back pain, and at times with illnesses unrelated to SCD None of the subjects exhibited the asthenic, eunuchoid habitus thought to be typical of homozygous sickle cell disease among adolescent and adult Negroes None of the

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patients had ulcerations of the lower extremities. A past history of bone and joint pain was infrequently obtained, with characteristic involvement of the shoulders and hips. Bouts of abdominal pain were relatively uncommon, and hemolytic or aregenerative crises were rare. By far the most consistent, major physical abnormality was the presence of splenomegaly, which was observed in 36 subjects. Hepatomegaly was noted in 20 subjects. Scleral icterus was not infrequent, but cutaneous icterus was seldom observed in the absence of intercurrent, acute liver disease.

Complications directly attributable to SCD were unusual. Two patients experienced thromboembolic episodes, one of whom expired presumably as the result of a massive pulmonary embolism. Intercurrent diseases possibly related to hemoglobinopathy and hemolytic disease included one instance of thrombocytopenia, a case of paroxysmal nocturnal hemoglobinuria reported in detail elsewhere [17], one patient with recurrent, gross hematuria, and 2 patients with cholelithiasis. There have been 3 deaths: one in a 32-year-old male with the nephrotic syndrome and terminal renal insufficiency, another in a 25-year-old female cited previously, who apparently succumbed to a pulmonary embolism, and a third death from unknown causes in a 32-year-old woman.

Abnormal chest X-rays were observed in 15 of 30 subjects having this examination performed. In 6, cardiac enlargement was observed and in 9, a variety of pulmonary lesions were seen which, with one exception could have been associated with underlying SCD. The most frequent abnormalities were nonspecific pulmonary infiltrates and accentuated bronchovascular markings. Skeletal X-rays were obtained in 36 subjects, most often in reference to episodes of bone and joint pain. The vertebrae were *frequently shown to be biconcave*, and together with the bony pelvis usually showed increased trabeculation and osteoporosis. The proximal long bones often showed cystic changes, with sclerotic zones in and adjacent to the humeral and femoral heads. Finally, the presence of widened cranial diploe in skull films was characteristic of the group.

Abdominal X-rays were obtained in 15 subjects, either as part of a skeletal survey, or in the course of gastrointestinal or pyelographic examination. Eight of these subjects demonstrated splenic opacities either in the form of nodularity or confluent calcific densities (fig. 1). The nature of these changes is not apparent, but presumably could

Table II Miscellaneous hematologic findings among Saudi Arabs with sickle cell disease

	No	Range	Mean	SD
MCV, μm^3	37	61–107	87.0	±12.9
MCH, pg	38	14–35	28.0	± 4.5
MCHC, %	52	24–36	34.0	± 3.1
Fetal hemoglobin, %	62	1.4–24.7	10.7	± 5.7
RBC osmotic resistance, % NaCl	26			
Beginning hemolysis		0.46–0.28	0.39	± 0.05
Complete hemolysis		0.34–0.0	0.26	± 0.04

RBC morphology	65 subjects
Anisocytosis	13
Poikilocytosis	11
Hypochromia	27
Target cells	14
Elliptocytosis	2

represent calcification of splenic infarcts, gross hemosiderin deposits, or both

There was considerable individual variation in hemoglobin concentrations, and a wide range of hemoglobin concentrations and red cell indices for the group Abnormal red cell morphology was frequently observed in the blood smears Red cell osmotic resistance was increased in 24 of 26 subjects tested Hemoglobin electrophoretic [illegible]

[illegible] in 9 of 15 subjects Seven of the 9 subjects with elevated A_2 hemoglobin had no detectable hemoglobin A on electrophoresis A mild to moderate reticulocytosis and a slight elevation of serum bilirubin were consistent findings among all patients tested [illegible]

[illegible] new and unrelated families, were found to have sickle cell disease Clinical and hematologic studies are incomplete, but hemoglobin electrophoresis suggests that many of these subjects are examples of sickle cell β-thalassemia



Fig 1 Abdominal X ray of 39 year-old Saudi ARAMCO employee with sickle cell disease and hepato-splenomegaly Hemoglobin electrophoresis S 80.1%, F 14.9%, A_2 5.0%

disease 19 showed an S pattern with increased A_2 hemoglobin, 13 showed an S >A pattern, and the remainder showed an S pattern with normal levels of A_2

Discussion

Sickle cell disease has been reported infrequently from the Middle East In 1956, AKSOY reported a series of 15 cases from Southern

Turkey, among a group of Eti-Turks [1]. Ethnically, this is an Arab group, and likely to have descended from Syrian or Egyptian immigrants. In many ways AKSOY's series resembled the group of Saudis discussed in this report. Splenomegaly was observed in 11 of 15 subjects, skeletal X-ray changes were minimal, target cells were conspicuous in the blood films of 10 subjects, and all patients demonstrated increased red cell osmotic resistance. In 1962, SHAHID and ABU HAYDAR [19] cited isolated case reports from Egypt, and added 3 cases of their own, two from Lebanon and one from Syria. BAKIR and AL-QAYSI reported the first case of SCD from Iraq in 1964 [3].

Traditionally, homozygous SCD has been regarded as an extremely debilitating disease, often fatal in childhood [21]. The diagnosis of SCD with mild manifestations in an adult immediately raises the question of possible heterozygosity for the sickle cell trait and another hemoglobinopathy such as hemoglobin C, D, or E. Of perhaps greater significance within Caucasian populations of the Middle East, is the possibility that sickle cell thalassemia may be present in individuals

SCD apparently does occur, and has been described in 60 Jamaican adults in a recent publication [18].

Among Saudi Arabs, it appears likely that many of the reported cases represent double heterozygosity for the sickle cell trait and another hemoglobinopathy. The occurrence of the S>A hemoglobin electrophoretic pattern in Saudis indicates that the usual type of sickle cell β-thalassemia disease is found in this population. In addition, the presence of increased A_2 hemoglobin in blood samples from many individuals with an S hemoglobin electrophoretic pattern suggests that sickle cell β-thalassemia, with virtually no production of hemoglobin A, may be the most frequent type of sickle cell disease encountered among adults.

Surprisingly, documented cases of thalassemia major are seldom encountered in the Saudi oasis population, from which the cases of sickle cell d

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zygous SCD. This would account for an aggregation of cases of sickle cell-thalassemia among adolescents and adults. Unfortunately, a survey for the presence of the β-thalassemia traits has not been attempted in Saudi Arabia.

Recent recognition of hemoglobin H disease and the presence of the α-thalassemia trait within the Saudi oasis population [16] indicates that double heterozygotes for the sickle cell trait and α-thalassemia can be expected. Although it has been assumed that no interaction occurs between α- and β-chain hemoglobinopathies, there is evidence that the presence of the α-thalassemia trait suppresses hemoglobin S formation in the double heterozygote [22]. This has given rise to speculation that α-chains combine preferentially with β^A-chains in the presence of β^S-chains. A possible extension of this hypothesis to include preferential combination of α with γ over α with β^S-chains in instances of homozygous sickle cell α-thalassemia disease would lead to the inference that relatively high levels of fetal hemoglobin could be expected in such cases. The pattern of hemoglobin components might thus be indistinguishable from that found in certain instances of sickle cell β-thalassemia disease, with no detectable hemoglobin A, and with elevated hemoglobin F. AKSOY [2] described this phenomenon in a case report of a 4½-year-old Turkish boy with clinical evidence of moderately severe SCD, striking abnormal morphology of the red cells, and a fetal hemoglobin of 27%. Studies of the parents revealed the presence of the sickle cell trait in the father, and sickle cell α-thalassemia in the mother.

There remains the possibility that a few cases of SCD in Saudis represent sickle cell-D-or sickle cell-G-disease. However, discrepancies between the results of sickling preparations and the appearance of S hemoglobin on electrophoresis have not been observed in a large number of routine laboratory investigations on Saudis over the past ten years, nor in the survey for the sickle cell trait cited previously [13], so that a combination of S-D or S-G hemoglobins would appear extremely unlikely.

That the occurrence of sickle cell-hemoglobin C-disease among the group of Saudis discussed in this report is not fortuitous is suggested by the 4 cases from separate families encountered in a group of 65 subjects with SCD. *There is no doubt that there has been a continuous* infusion of African genes into the East Arabian populations for centuries as a result of the extensive slave-trading operations in the Persian

Gulf [10] Although LEHMANN [12] has suggested that the sickle cell trait originated in Arabia and spread to Africa the reverse is more likely to have been the case

High levels of alkali resistant hemoglobin which have been observed in some cases of SCD among Saudis could be the result of interaction between the sickle cell trait and hereditary persistence of fetal hemo globin However, the S F heterozygote is characteristically free from the stigmata which accompany homozygous sickle cell disease and sickle cell thalassemia Moreover, the electrophoretic pattern shows absence of hemoglobin A and normal amounts of hemoglobin A_2 Presumably the gene for hereditary persistence of fetal hemoglobin originated in Africa [4] but a similar if not identical trait has been reported in several Greek families [7] Considering the proximity of the

Several isolated instances of unexplained elevation of fetal hemoglobin in otherwise normal adult Saudis have been observed, however, these have not been verified by appropriate follow up and repeated deter minations of alkali resistant hemoglobin

LEWIS [14] and LEWIS *et al* [15] have offered the interesting hypo thesis that the presence of erythrocyte glucose 6-phosphate dehydro-genase (G 6-PD) deficiency may in some way mitigate full expression of homozygous SCD and have shown that Ghanian subjects with the sickle cell trait show a significantly higher frequency of G 6 PD deficiency than controls with only hemoglobin A and the frequency is yet higher among subjects with SCD A similar situation prevails in the Saudi oasis population where approximately 60% of male subjects with the sickle cell trait have been observed to have G-6-PD deficiency [9 10] Study of this association among Saudis has not been extended to include a large number of subjects with SCD but the possibility of interaction between hemoglobin S and G 6-PD deficiency might ideally be assessed in a population with a high frequency of both defects and in which the Caucasian G 6-PD mutant is present

One of the interesting biproducts of the reported study has been the demonstration of calcific densities of the spleen in a high propor tion of cases in which abdominal X rays were available HENLEY *et al* [11] reported a series of 9 cases of SCD in which distinctive, punctate

densities were seen in the spleen on radiologic examination In the Saudi cases the splenic densities tended to be conglomerates rather than miliary opacities, and all had palpable spleens on physical examination It is possible that the disparate radiologic pictures represent two different phenomena, and that in the Saudi group with splenic opacities and splenomegaly, the process is that of massive hemosiderosis Because of the well-known tendency of adolescents and adults with homozygous SCD to develop splenic atrophy, or actually undergo autosplenectomy, it appears likely that the 'opaque spleen syndrome' indicates a SCD variant rather than homozygous SCD

The skeletal X-ray findings in Saudi subjects with SCD are not distinctive they have been described in patients with homozygous disease and SCD variants, and represent varying degrees of bone marrow hyperplasia and the cumulative effects of multiple bone infarcts

In conclusion, it appears that SCD is frequent among the oasis Saudi Arab population, that a considerable proportion of Saudis with this condition survive into adult life, and that SCD in this group is a heterogeneous assortment of at least 4 entities homozygous SCD, two types of sickle cell β-thalassemia, and hemoglobin S-C disease.

Summary

A review of clinical and hematologic findings in a group of 65 adult and adolescent Saudi Arabs with sickle cell disease has been presented with additional data on hemoglobin electrophoretic patterns encountered in another group of 76 incompletely studied subjects with sickle cell disease This study indicates that sickle cell β thalassemia accounts for a considerable proportion of all cases of sickle cell disease in the Saudi oasis population and is usually characterized with hemoglobin electrophoresis by absence of hemoglobin A and elevated hemoglobin A_2 Four cases of hemoglobin S-C disease were encountered in the study The implications of hypothetical interaction between hemoglobin S α thalassemia hereditary persistence of fetal hemoglobin and G-6-PD deficiency are discussed

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A Ghanaian Adult, Homozygous for Hereditary Persistence of Foetal Haemoglobin and Heterozygous for Elliptocytosis[1]

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The gene which maintains foetal haemoglobin after the neonatal period by a failure to switch from γ chain to β chain production was first described by EDINGTON and LEHMANN in 1955 in Ghana [1], and the description fully reviewed by THOMPSON and LEHMANN in 1962 [2] This abnormality has been discovered in Uganda by JACOB and RAPER [3], in Jamaica by WENT and MACIVER [4], in the United States by BRADLEY and CONLEY [5], OLIVIA and MYERSON [6], RUCKNAGEL and NEEL [7], KRAUS *et al* [8], in Greece by FESSAS *et al* [9] and in Italy by MANGANELLI *et al* [10] All the published cases except two records, were heterozygotes One of the homozygotes first described by WHEELER and KREVENS [11] and reviewed by CONLEY *et al* [12] in 1963 was an American Negro child The other report came from Italy, published by POLOSA *et al* [13] on two Caucasian siblings, who were 4 years and 3 years respectively at the time of publication, i e in 1964

Homozygosity is very uncommon among the rare haemoglobin carriers because the probability of pairing is low, it is important, therefore, to exclude every possible factor which might disguise hidden heterozygosity, notably thalassaemia and the high F gene In this paper we present a family where the father is the first described adult homozygous carrier for hereditary persistence of foetal haemoglobin

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and in addition, he has another red cell anomaly: elliptocytosis. All of the children inherited the former abnormality in a heterozygous state and two of them also have elliptocytosis.

Methods

Haemoglobin electrophoresis was carried out on filter paper with tris buffer at pH 8.9, agar gel electrophoresis with citrate-citric acid buffer, pH 6.0, starch gel electrophoresis with tris-citrate and borate buffer pH 8.6. Hb F was measured as alkali resistant haemoglobin according to SINGER *et al* [14], Hb A_2 was determined by the method of MARENGO-ROWE [15]. To demonstrate foetal haemoglobin in red cells KLEIHAUER and BETKE's method was used [16]. G-6PD determination was carried out according to the description of BREWER *et al* [17], osmotic fragility according to PARPART *et al* [18]. Red cell survival time was measured by the method given by LAJTHA [19]. The haemoglobin of the propositus was examined by 'finger printing' of the tryptic peptides by the methods summarized previously [20].

Case History

A 45-year-old Ghanaian male came to the Korle Bu Teaching Hospital, Accra, with the complaints of numbness of his right side of the body and burning and soreness of the right palm. He had been perfectly well all his life and there was no history of serious injury. He was married with two wives and had 10 children (5 of whom were available for investigation). On examination he was overweight 173 lb. at 5' 4"). There were no objective neurological signs in the peripheral nervous system and no positive findings in the chest. Spleen 6 cm below costal margin, liver not palpable. The right arm was held rigidly with slight limitation of movement at shoulder joint due to pain. The right wrist and hand were swollen with limitation of movement. The right hand was definitely warmer than the left and the skin on his hand and forearm was shiny.

Investigation PCV 43% Hb 15.4 g [illegible]

Management and progress: gout was diagnosed and patient was put on Probenecid 500 mg daily, with some clinical and laboratory improvement.

Family Study

Ago B. (II.1), 21 years and eldest son, did not have any physical signs. Bow B. (II.2), 14 years, spleen 5 cm below costal margin. Liver is not palpable. [illegible] B. (II.3), [illegible] years, has slightly enlarged spleen, 2 cm below costal margin and the liver 2 cm below costal margin. Afote B. (II.4), who is a step brother, has an enlarged liver of which the edge is palpable 7 cm below costal margin, the spleen is also palpable, 8 cm below the rib. Asedu B. (II.5), 8 years, has a soft precordial bruit with accentuation of pulmonic second sound.

Table I

Name	Code No	Age years	Position in the family	PCV %	Hb g %	Retics %	WBC/ml	Red cell morphology
Boye, B Snr	I 1	46	father (propositus)	43	15 4	5 3	5 900	Elliptocytosis, aniso-poikilocytosis aniso-chromia, target cells
Odatsuro	I 2	42	mother	42		0 2		Normocytosis normochromia
Ago, B	II 1	21	son	45	15 0	3 2	7 800	Normocytosis normochromia
Boye, B Jr	II.2	14	son	36	12 0	2 0	6 700	Normocytosis normochromia
Ashong, B	II 3	10	son	36	11 6	3 2	6 100	Elliptocytosis normochromia
Afote, B	II 4	9	son[1]	36	12 0	0 4	5 800	Normocytosis normochromia
Asiedu, B	II 5	8	son	35	11 6	0 8	5 900	Elliptocytosis normochromia

[1] Different mother

Laboratory Studies

The patient (I 1) had no anaemia, the reticulum cell count was slightly increased more than 80% of his cells were elliptocytes Many of these cells showed a regular elliptical form, but some of them were elongated with disproportionate longitudinal axis There was anisocytosis, some elliptical cells had a bend on one end Small cells and cells with triangular shape could also be seen Anisochromasia and occasional target cells could also be observed (fig 1) Serum iron and TIBC were within normal limits Red cell survival time (T^{1}_{2}) was 17 days (our normal values 26–39 days) Osmotic fragility of the red cells was slightly decreased (fig 2)

None of the children of the propositus was anaemic, although II 3, 4 and 5 had slightly lower Hb concentration and 3 had a reticulocyte count above 1% Two of the children had elliptocytosis but without anisopoikilocytosis or anisochromasia The elliptocytes had a rather regular shape and target cells were absent The osmotic fragility of the propositus' red cells was slightly decreased, all the other samples showed normal fragility

The sickling test in all samples was negative Using the KLEIHAUER and BETKE technique there was no elution of haemoglobin from the red cells of the propositus using acidic buffer (fig 3) With the same technique some haemoglobin was eluted from the red cells of the children, the cells were 'half empty' but the distribution was uniform all cells mormocytes and elliptocytes contained a certain amount of haemoglobin The foetal haemoglobin concentration in the blood of the propositus was 87% by the alkali denaturation technique

Table I (continued)

Hb A	*Hb F* %	*Hb A_2* %	Sickling test	KLEIHAUER BETKE test	*G-6PD*	*Hb type*
0	87*	0	negative	+ + + +	normal	FF high gene
+ + + +	1.8	2.[illegible]	negative	negative	defect	AA
+ +	26	2.7	negative	+ +	defect	AF high gene
+ +	28	2.7	negative	+ +	defect	AF high gene
+ +	27	2.7	negative	+ +	defect	AF high gene
+ +	20	2.6	negative	+ +	defect	AF high gene
+ +	25	2.6	negative	+ +	defect	AF high gene

* At very high concentration the measurement of Hb F by alkali resistance gives erroneously low results by other methods Hb F % was 100

which gives erroneously low results at high concentration of Hb F In the wife's blood Hb F was below 2% and in the children's between 20 and 28%. Hb A and A_2 were totally absent from the propositus blood as demonstrated particularly on agar electrophoresis for A, and paper electrophoresis for A_2, however the blood of the children contained both. By electrophoresis on filter paper the blood of the propositus showed only one thick band and this was *situated between the start line and the usual position of Hb A* near to the latter (fig 4) On starch gel there was also a single line slightly beyond the Hb A. Neither on starch nor on paper was Hb A visible On agar pH 6.0 there was a single thick spot ahead of Hb A. The blood of his children had 2 bands one thinner band on the same level where their father's single line was located, and *another thicker band* corresponding to their mother's Hb A (fig 5) G-6PD content of the propositus blood was normal but all the five children had a defect as did the wife (mother of II 1, 2 3 and 5 Fingerprint of the tryptic digest of the propositus haemolysate showed only the peptides of foetal haemoglobin (fig 6

Discussion

The diagnosis of the homozygous state for hereditary persistence of foetal haemoglobin in our case is based a) on the high concentration



Fig. 1. Red blood cells of the propositus, Leishman staining. Elliptocytes, aniso-poikilocytosis, anisochromia and one target cell.

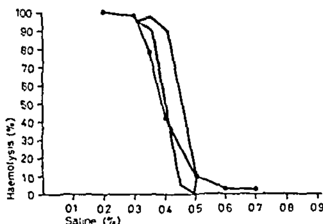


Fig. 2 Red cell fragility curve, propositus.

of Hb F, b) the absence of Hb A and A_2, c) the even distribution of acid fast haemoglobin in the red cells, d) on family study: the propositus has only Hb F, his wife Hb A and all the children have both A and F, the latter in high concentration, e) on the result of fingerprinting of the haemoglobin of the propositus.

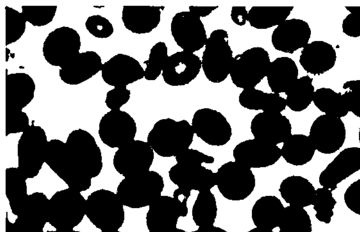


Fig 3 Blood film propositus Kleihauer Betke technique The cells are filled with haemoglobin no elution

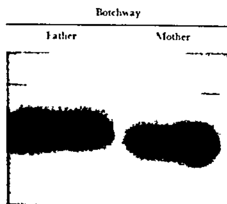
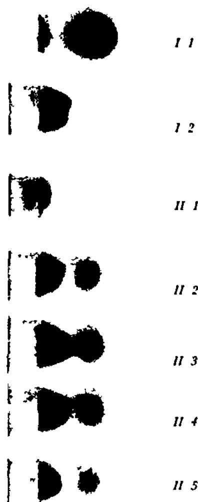


Fig 4 Paper electrophoresis, pH 8.9 The propositus blood (left) shows one single thick band (Hb F which is nearer to the starting point than his wife's (Hb A) (right) The former does not contain Hb A_2 which could be seen in the latter's blood as a faint band

The propositus has also another inherited abnormality: elliptocytosis The family study has revealed that the father has passed it on to two of his children (fig 7) The father must therefore be a heterozygote The genes for elliptocytosis and hereditary persistence of foetal haemoglobin are non-allelic and there does not seem to be



any linkage between them However, study of some other characters may further clarify this point The propositus' elliptocytes were anisocytotic and showed anisochromasia with occasional target cells. The elliptocytes of the two children were rather regular and target cells were absent This morphological alteration could be due to the high concentration of foetal haemoglobin and the different way of organization of Hb F in the cell [12].

Because of the combination of hereditary persistence of foetal

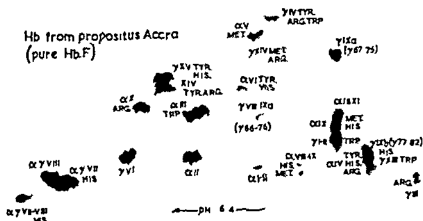


Fig. 6. Fingerprint of the haemoglobin of the propositus. There is only haemoglobin F present.

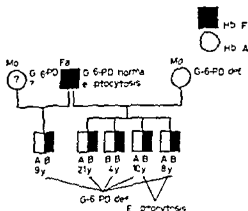


Fig. 7. Family tree showing homozygosity for high F and heterozygosity for elliptocytosis of the propositus.

haemoglobin and elliptocytosis it is difficult to compare our findings with those of the other 3 individuals described with homozygous high F (table II). The comparison seems to show that there are some common features. The carrier is not retarded in his mental or physical development, clinical signs of haemolysis are very slight or absent, moderate enlargement of the spleen may or may not be present. In the laboratory findings anaemia is rather uncommon, the osmotic

Table II

Author, year of publication	Age	PCV %	Hb g%	Retics %	Hb F %	Hb A_2 %	Osmotic fragility
WHEELER and KREVANS, 1961 [11] revised by CONLEY *et al.*, 1963 [12]	33 months	41	13.3	0.6	87–92	0	decreased
POLOSA *et al.*, 1964 [13]	4 years	–	8.8	1.8	100	0	decreased
POLOSA *et al.*, 1964 [13]	3 years	–	11	–	100	0	decreased
Our case	45 years	43	15.4	5.3	87[1]	0	decreased

[1] See [1] table I

fragility is slightly decreased and the red cell survival, determined only in our case, was slightly shortened. There is irregularity in the shape of the red cells with a moderate number of target cells; the red cells contain acid fast haemoglobin, and Hb A and A_2 are absent from the blood. The fingerprinting in our case and in one of the others showed only Hb F and this was not different from the Hb F which is in the umbilical blood.

Our case demonstrates that man can reach the fifth decade in a good physical and mental condition with the haemoglobin of a foetus circulating in his body.

Summary

Laboratory and family studies confirmed the diagnosis of homozygosity for hereditary persistence of foetal haemoglobin and heterozygosity for elliptocytosis in a 45-year-old Ghanaian male. This is the first adult where the homozygous state was found and showed that man can reach the middle age in excellent physical and mental condition with the haemoglobin of a foetus.

Table II (continued)

Spleen enlarge-ment	Liver enlarge-ment	Red cell morphology	Signs of haemolysis		Red cell survival	Finger printing
			clinical	*laboratory*		
0	0	Aniso-poikilo-cytosis target cells, cells resembling spherocytes	0	0	0	normal foetal Hb
+ +	0	Anisocytosis, hypochromia, anulocytes 'pyriform cells'	+	0	0	–
0	+ +	Aniso-poikilo-cytosis anulocytes	+	0	0	–
+ +	0	Elliptocytosis, aniso-poikilo-cytosis, anisochromia, target cells	0	+	17 days*	normal foetal Hb

* Our normal 26–39 days.

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Tumorartige intrathorakale extramedulläre Hämatopoese bei hämolytischer Anämie[1]

Bericht über einen Fall und Literaturübersicht

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Hämatologische Abteilung und Röntgen Abteilung der Medizinischen Klinik und Poliklinik (Geschäftsführender Direktor Prof Dr O H Arnold) und Pathologisches Institut (Direktor Prof Dr W Müller) des Klinikum Essen der Ruhr Universität

Extramedulläre Hämatopoese wird vor allem bei ausgedehnten Knochenmarkveränderungen durch Osteomyelosklerose bzw Myelofibrose, Marmorknochenkrankheit Albers Schönberg, Leukämien, Karzinosen oder Lymphogranulomatose [26, 30], in einzelnen Fällen bei Osteodystrophia fibrosa generalisata Recklinghausen [31], Rachitis [15] und Ostitis deformans Paget [9] beobachtet Ferner tritt sie bei Perniciosa, fetaler Erythroblastose sowie bei akuten Infektionen auf [26 30] Sie findet im allgemeinen in Milz, Leber und Lymphknoten, gelegentlich in Dura mater, Thymus, Nebennieren, Prostata, Mamma, Parametrien oder organisierten Thromben statt [5, 30] und führt zu einer meist diffusen Infiltration dieser Organe Das Auftreten tumorförmiger extramedullärer Blutbildungsherde im Bereich der Nierenhilı [5, 14], im kleinen Becken [4, 9, 20] und im Thorax gilt dagegen als ungewöhnlich Die intrathorakale Knochenmarkheterotopie scheint nach der vorliegenden Literatur vorwiegend bei angeborenen hämolytischen Anämien vorzukommen Es wird im folgenden über eigene Beobachtungen bei einer Patientin mit Kugelzellenanämie berichtet

Kasuistik

Pat G H, geb 5 1 1910 Hausfrau wurde im April 196[illegible] zum erstenmal in unsere Klinik aufgenommen Anamnestisch bestand 19[illegible]2 vorübergehend ein Ikterus ohne sonstige

[1] Herrn Professor Dr O H Arnold zum 60 Geburtstag in Dankbarkeit gewidmet.



Abb 1 Thoraxaufnahme (*a* p a *b* li anl) Grosse weichteildichte Verschattungen bds paravertebral

Symptome Seit 1937 war eine Anämie bekannt, die nach den Angaben der Patientin als Perniciosa gedeutet und mit Leber bzw Vitamin B_{12} Praparaten behandelt wurde

Zur Familienanamnese war zu erfahren, dass der Vater der Patientin zeitlebens an einer Blutarmut litt und durch eine Gelbfärbung von Gesicht und Skleren auffiel Er sollte deswegen splenektomiert werden lehnte die Operation jedoch ab Er starb mit 71 Jahren an einer Lungenembolie Bei dem Sohn der Patientin traten bereits in früher Kindheit eine ausgepragte Anämie und ein Ikterus auf die angeblich durch einen verstärkten Blutzerfall bedingt waren Die Milzexstirpation im Alter von 7 Jahren führte zu einer prompten Besserung Seitdem war er vollig beschwerdefrei ein Ikterus trat nicht mehr auf Da er seit mehreren Jahren als Monteur im Ausland tätig ist konnten Blutuntersuchungen nicht durchgefuhrt werden

Es bestanden bei der Patientin eine asthmoide Emphysembronchitis ein Subikterus und eine Cholelithiasis Die Milz war 3 cm unterhalb des Rippenbogens tastbar und von derber Konsistenz Die Thoraxaufnahme zeigte weichteildichte homogene Verschattungen beiderseits paravertebral rechts in Hohe von D 7 bis D 10 links in Hohe von D 9 bis D 11 (Abb. 1) Durch die Bronchoskopie und Mediastinoskopie konnte eine Artdiagnose dieser Tumoren nicht gestellt werden[1] Auf eine Punktion oder eine Thorakotomie musste wegen der hochgradig eingeschränkten Lungenfunktion verzichtet werden

Laborbefunde Hamoglobin 11 3 g 100 ml Erythrozyten 3 8 Mill mm³ Hb$_E$ 29 pg Hämatokrit 33% mittlere Hämoglobinkonzentration des Einzelerythrozyten 34 2% mittlerer Erythrozytendurchmesser 6 4 μm mittlere Erythrozytendicke 2 5 μm mittleres Erythrozyteneinzelvolumen 86 8 μm^3 sphärischer Index 0 40 Im Blutausstrich reichlich kleine, *hämoglobinreiche Erythrozyten ohne zentrale Aufhellung (Sphärozyten)*, Anisozytose Da der mittlere Erythrozytendurchmesser vermindert und die mittlere Erythro-

[1] Fur die Durchführung dieser Untersuchungen danken wir Herrn Priv Doz. Dr W MAASSEN Chefarzt der Ruhrlandklinik der LVA Rheinprovinz, Essen Heidhausen



Abb. 7 Histologisches B ld des Tumorgewebes m t Megakaryozyten erythropoetischen und gran lopoet sc en Vorstufen (Hämat xyl n Eos n × 1?

zytend cke erh ol t waren ergab s ch ein erh d ter spl änischer Index der im Zusammenhang m t dem normalen Ery!l rozyteneinzel olu nen für das Vorl egen von Kugelzellen sprach Leukozyten ?t 400 m n³ D ff rentialt lutb ld Promyelozyten 2% Myelozyten ?% Metam elozyten ??% Stabkern ge 22% Segm entkern ge 60% Lymphozyten 3% Monozyten 4° Normoblasten 7 Ret kul ozyten ? ‰ Thrombozyten ?60 000 mm³ Osmot sche Resistenz der Erythrozyten 0,?–0 40% NaCl B l rul n d rekt Spur ind rekt 2 0 mg/ 100 ml. LDH 96 mU ml SCOT 7,2 mU m l SGPT 2 7 m U ml. D rekter ind rekter und Super Coombs-Test negat v BKS 51/?0 mm n W S ernalmark Hyperplase der Erythropoese m t Vermehrung der kernhal gen reifen Vorstufen leicht l nksverschobene Granulopoese

Aufgrund der Fam l en und Eigenanamnese sowie der bei der Pat entin erhobenen Blutbefunde wurde d e Diag se einer wahrsche nl ch angeborenen Kugelzellenanäm e gestellt

Na h Therapie m t Digox n Te racycl n und Expektorantien wurde die Patientin ent lassen eine Splenektom e lehnte sie ab. Laborbefunde bei der En lassung Hämoglobin 11 ? g 100 m l Erythrozyten 3 9 M ? mm³ Leukozyten 10 400 mm³ Differentialblutbild Stabkern ge 3%, Segmentkernige 81%, Basoph le 1%, Lymphozyten 13%, Monozyten ?%, Ret kulozyten 94‰ Bil rubin d rekt nega v ind rekt ? ? mg 100 ml. LDH 16? mU ml BKS ??0 mm n.W

Im Januar 19?? erfolgte d e erneu e Einweisung wegen einer Exazerbation der seit Jahren bestehenden schweren a l mosalen Empl sem bronch s und e ner Rechtsherz

insuffizienz. Die Thoraxaufnahme liess eine geringe Grössenzunahme der Tumoren erkennen.

Laborbefunde: Hämoglobin 10,8 g/100 ml, Erythrozyten 3,3 Mill./mm³, Hb_E 32,5 pg, Hämatokrit 32%; Leukozyten 13 000/mm³, Differentialblutbild: Promyelozyten 1%, Stabkernige 21%, Segmentkernige 69%, Basophile 1%, Eosinophile 2%, Lymphozyten 4%, Monozyten 2%, Normoblasten 4; Retikulozyten 13‰. Bilirubin: direkt Spur, indirekt 1,4 mg/100 ml. LDH 42 mU/ml. BKS 92/125 mm n.W.

Trotz Therapie mit Digoxin, Tetracyclin, Expektorantien und intermittierender O_2-Zufuhr verstarb die Patientin nach vier Tagen unter den Zeichen des akuten Rechtsherzversagens. Als wesentlicher pathogenetischer Faktor der kardialen Insuffizienz dürfte die schwere Lungenfunktionsstörung (obstruktive Ventilationsstörung) bei ausgeprägtem Lungenemphysem anzusehen sein. Eine Verschlimmerung des Leidens infolge einer zusätzlichen, durch die Tumoren bedingten restriktiven Ventilationsstörung war allerdings nicht sicher auszuschliessen. Eine Lungenembolie lag nicht vor. Die paravertebral, retropleural gelegenen Tumoren wiesen makroskopisch eine multilobuläre Anordnung auf. Sie bestanden aus einer dunkelroten, weichen Gewebsmasse. Bei der histologischen Untersuchung fielen in dem zellreichen, polymorphen und gefässhaltigen Gewebe immer wieder Megakaryozyten auf (Abb. 2). Ausserdem fanden sich Erythrozyten, Normoblasten, segmentkernige Granulozyten und granulopoetische Vorstufen sowie Plasmazellen und vereinzelt Gewebsbasophile. In dem teilweise septenartig verdichteten Bindegewebe war reichlich eisenpositives Pigment abgelagert.

Diskussion

Intrathorakale Tumoren als seltene Manifestation einer extramedullären Hämatopoese treten vorwiegend bei angeborenen hämolytischen Anämien auf, z. B. bei Kugelzellenanämie, Thalassämie und Sichelzellenanämie (Tab. I). Bei erworbenen hämolytischen Anämien sind sie dagegen bisher nicht beobachtet worden. Auch bei unserer Patientin bestanden tumorartige Veränderungen im hinteren unteren Mediastinum bei einer wahrscheinlich angeborenen hämolytischen Anämie (Kugelzellenanämie). Da histologisch in diesen Tumoren Megakaryozyten, sowie erythropoetische und granulopoetische Vorstufen nachgewiesen wurden, besteht kein Zweifel daran, dass es sich um extramedulläre Blutbildungsherde handelte. Die ausgeprägte Ablagerung von eisenpositivem Pigment kann als Folge rezidivierender Blutungen und/oder einer gesteigerten Hämolyse in diesem Gewebe interpretiert werden. Für eine extramedulläre Hämatopoese sprach auch die im peripheren Blut mehrfach beobachtete pathologische Linksverschiebung [26].

Inzwischen wurde eine zweite Patientin mit einem raumfordernden tumorartigen Prozess im hinteren Mediastinum beobachtet, bei der ebenfalls eine hämolytische Anämie (Kugelzellenanämie) vorliegt. Obwohl röntgenologisch eine auffallende Ähnlichkeit mit dem bei der ersten Patientin nachgewiesenen Mediastinaltumor besteht, kann die Diagnose eines extramedullären Blutbildungsherdes nicht gestellt werden, da eine histologische Untersuchung bislang nicht möglich war.

Die blutbildenden intrathorakalen Tumoren liegen fast immer beiderseits paravertebral und kommen nur selten oberhalb des 5 Brustwirbelkörpers vor (Tab 1) Sie sind rundlich und glatt begrenzt und besitzen eine multilobuläre Anordnung Eine Verbindung zu den benachbarten Knochen besteht nicht Im allgemeinen verursachen sie keine klinischen Symptome Meist treten sie erst nach dem 50 Lebensjahr auf, doch sind sie vereinzelt auch bei jungeren Patienten beobachtet worden Ihre Abgrenzung gegenuber anderen raumfordernden Prozessen im hinteren Mediastinum oder in den paravertebralen Lungenabschnitten ist allein durch die Rontgen Untersuchung nicht sicher moglich Differentialdiagnostisch kommen vor allem neurogene Tumoren (Neurinome), Malignome (Bronchialkarzinome, Pleuraendotheliome, Metastasen), Lymphome, Mischgeschwülste (Teratome, enterogene und bronchogene Zysten) und Senkungsabszesse in Betracht [1, 16] Bei unserer zweiten Patientin sprechen die szintigraphischen Untersuchungen gegen das Vorliegen eines Lungentumors oder einer intrathorakalen Nebenmilz Ein blutbildender intrathorakaler Tumor sollte immer dann in Erwägung gezogen werden, wenn eine angeborene hämolytische Anämie besteht

Die extramedullare Hämatopoese wird von den meisten Autoren als Kompensationszeichen angesehen, wenn der Zellbedarf gesteigert oder die Knochenmarkfunktion eingeschrankt ist Die Pathogenese der intrathorakalen tumorförmigen Blutbildungsherde ist bisher noch nicht geklärt [2, 3, 4, 5, 7, 13, 26, 30] Sie werden einerseits auf eine metastatische Absiedlung von Knochenmarkzellen, andererseits auf eine Metaplasie von Resten der fetalen Blutbildung zuruckgeführt Ferner wird eine embryonale Keimversprengung diskutiert, die infolge der allgemeinen Aktivitätssteigerung des hämatopoetischen Systems zur Blutbildung angeregt wird Für die in den Nierenhili und im präsakralen Gewebe nachgewiesenen extramedullären Blutbildungsherde wurde ebenfalls eine autochthone Entstehung durch Metaplasie von potentiell hämatopoetisch aktiven Zellen angenommen Im Gegensatz dazu findet unseres Wissens im hinteren Mediastinum auch während der fetalen Blutbildungsperiode keine Hamatopoese statt Es müssten somit mesenchymale Zellen (Adventitiazellen, Histiozyten) als mögliche hämatopoetische Vorstufen angenommen werden Dennoch bleibt bisher unverständlich, warum derartige Tumoren vorwiegend bei hämolytischen Anämien und nur selten bei anderen

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Laborbefunde Hamoglobin 10 8 g/100 n
Hämatokrit 32%, Leukozyten 13 000 mr
Stabkernige 21% Segmentkernige 69% 1
4% Monozyten 2% Normoblasten 4 1
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Trotz Therapie mit Digoxin, Tetracycl
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Tabelle I (Fortsetzung)

Nr.	Autor		Alter Jahre	Geschlecht m/w	Diagnose	Lokalisation der Tumoren
14	Hanford R. B. *et al.* 1960	[12]	61	m	Kugelzellenanämie	bds. paravertebral D 5–D 12
15	Hanford R. B. *et al.* 1960	[12]	53	m	Verdacht auf Kugelzellenanämie, Gicht	links paravertebral D 8–D 12
16	Knoblich R. 1960	[17]	63	m	Thalassaemia minor	links paravertebral D 6–D 11
17	Litwer, H. 1960	[18]	63	m	Kugelzellenanämie	bds. paravertebral D 6–D12
18	Litwer, H. 1960	[18]	62	m	Kugelzellenanämie	bds. paravertebral unteres Mediastinum
19	Coventry W. D. *et al.*, 1960	[7]	50	m	Verdacht auf Kugelzellenanämie	bds. paravertebral D 10
20	Malamos, B. *et al.*, 1962	[21]	24	m	Thalassaemia major	bds. paravertebral D 2–D 10
21	Lowman R. M. *et al.*, 1963	[19]	43	m	Kugelzellenanämie	hinteres Mediastinum
22	Lowman, R. M. *et al.*, 1963	[19]	66	m	Kugelzellenanämie	hinteres Mediastinum
23	Papavasiliou C. *et al.* 1964	[23]	24	m	Thalassaemia major	bds. paravertebral
24	Papavasiliou C. *et al.* 1964	[23]	28	m	Thalassaemia major	rechts paravertebral unteres Mediastinum
25	Papavasiliou C. *et al.*, 1964	[23]	21	m	Thalassaemia major	rechts paravertebral
26	Sorsdahl, O. S. *et al.* 1964	[29]	40	m	Thalassaemia intermedia	links paravertebral D 9–D 10 extradural D 5–D 8
27	Sorsdahl, O. S. *et al.*, 1964	[29]	45	m	Thalassaemia intermedia	links paravertebral D 8–D 9
28	Seidler, R. C. *et al.*, 1964	[28]	31	m	Sichelzellenanämie	bds. paravertebral D 8–D 10
29	Condon, W. B. *et al.*, 1965	[6]	56	m	Kugelzellenanämie	bds. paravertebral hinteres Mediastinum

mit extramedullärer Blutbildung einhergehenden Erkrankungen beobachtet werden. Vielleicht liegt den Tumoren eine echte Missbildung zugrunde. Gezielte histologische und zytologische Untersuchungen an menschlichen Feten können möglicherweise zu einer Klärung dieser Fragen beitragen

Zusammenfassung

Es wird über eine Patientin mit Kugelzellenanämie berichtet, die tumorartige Veränderungen im hinteren unteren Mediastinum aufwies In dem Tumorgewebe konnten histologisch Megakaryozyten sowie erythropoetische und granulopoetische Vorstufen nachgewiesen werden Es handelte sich somit um extramedulläre Blutbildungsherde In der Literatur sind bisher 29 ähnliche Fälle veröffentlicht worden Die Pathogenese dieser intrathorakalen extramedullären Hämatopoese wird diskutiert

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Congenital Deficiency of Fibrinogen in Two Sisters

A Clinical and Haematological Study

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Congenital afibrinogenaemia is a rare coagulation anomaly. Since its first description by Rabe and Salomon [31] in 1920, about 60 cases have been added to the literature From this country, to our knowledge, three instances of this interesting disorder have been recorded [1, 20, 21]

Though death by excessive bleeding has been noted in the siblings of these patients in several reports [5, 17, 20, 22, 23, 32], proven afibrinogenaemia in the siblings has been recorded very infrequently [11, 24] The present communication describes the clinical and haematological features of this entity in two sisters

Material and Methods

Blood was collected by siliconized syringe in plastic tubes from an antecubical vein and mixed with 3.8% sodium citrate (1 part citrate to 9 parts blood) All the haematologic tests on coagulation unless otherwise stated were performed as described by Biggs and Macfarlane [3] Thrombin employed for determination of thrombin time was prepared according to Mellanby modified by Quick [30] Fibrinogen was estimated as described by Quick [30] The platelet count was done by direct counting using Thoma s pipette and improved Neubauer chamber Sodium citrate was used as diluent

The studies of platelet adhesion and aggregation were done in citrated platelet rich plasma with the use of phase contrast microscopy Viscous metamorphosis was studied under the following conditions in a plastic tube a mixture was made of equal parts of platelet rich plasma (PRP and M 40 calcium chloride or thrombin (1/1 000 dilution Mellanby) The tube was shaken for about 10 sec and a drop of mixture was transferred for a cover slip preparation which was studied intermittently for 4 h

Macroscopic examination of platelet aggregation with adenosinediphosphate (ADP) epinephrine nor epinephrine and thrombin was carried out at 37 C by adding 0.1 ml of these reagents to 0.2 ml of PRP

Reaction of platelets to kaolin was studied by the method of Hardisty et al [15]

Case Reports

Case 1 R., a Muslim girl, 7 years of age, developed a painful swelling of the gum during the eruption of her first permanent lower right molar tooth. Six days later she started bleeding profusely from this area which was uncontrollable. Next day she was admitted to Sir Sunder Lal Hospital, Banaras Hindu University.

On examination, severe bleeding was noticed from the gum. Bluish discoloration of skin, due to extravasation of blood underneath, was noticed on the right side of the neck, extending up to the clavicle. On interrogation it was revealed that she had displayed bleeding tendency since birth. She had notable umbilical bleeding for about 2 weeks, had easy bruising from mild injuries and her cuts bled for unduly long periods. There was no history of any spontaneous bleeding, nose bleeds or haemarthrosis.

After collection of blood for investigations, one bottle of fresh blood was transfused which effectively controlled the bleeding from the gums within 4 h.

Case 2 A., one of the elder sisters of R., aged 20 years and married, had similar complaints. She also had umbilical bleeding for about 12 days, had easy bruising and prolonged bleeding from cut injuries and at the time of fall of deciduous teeth. In addition, she has mild menorrhagia. One year back she had conceived but aborted at 3 months and had severe post-abortal uterine bleeding.

The pedigree is depicted in figure 1. The history of consanguinity is quite obvious. Fibrinogen levels less than 200 mg% were observed in both the parents who had one third

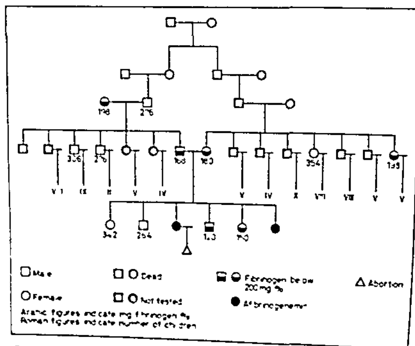


Fig. 1. Pedigree of afibrinogenaemia.

Table I Coagulation studies

Test	Case 1, 7 years	Case 2, 20 years
Whole blood clotting time	infinite (more than 6 h)	infinite (more than 6 h)
Kaolin cephalin clotting time	infinite (more than 6 h)	infinite (more than 6 h)
Prothrombin time	infinite (more than 6 h)	infinite (more than 6 h)
Thrombin time	infinite (more than 6 h)	infinite (more than 6 h)
Bleeding time *(Duke)*	Prolonged oozing from the site of venepuncture Next day after blood transfusion bleeding time was 6 min	Prolonged oozing from the site of venepuncture
Platelets	180,000/μl	230,000/μl
Prothrombin consumption Index	less than 10% (normal)	less than 10% (normal)
Thromboplastin generation	normal	normal
Thrombin generation	normal	normal

Table II Fibrinogen studies (cases 1 and 2)

a) Plasma heated to 60°C	No precipit
b) Plasma half saturated with NaCl	No precipit
c) Plasma quarter saturated with Amm sulphate	No precipit
d) Plasma + thrombin No clot	
e) Plasma electrophoresis No evidence of fibrinogen	

of children with afibrinogenaemia one third with fibrinogen levels less than 200 mg% and the remaining third with normal fibrinogen values

Coagulation studies Tables I and II show the results of coagulation studies done on both the sisters When to three parts of the patients plasma, one part of either fresh plasma old plasma aluminium hydroxide adsorbed plasma, haemophilic plasma or fibrinogen (Fluka) was added complete correction of kaolin cephalin clotting time and prothrombin time ensued The addition of fresh serum had no correcting effect

Studies on Platelets (Case 1)

Platelet aggregation Adhesiveness of platelets to glass was studied by direct observation of cover slip preparation under the phase contrast microscope Though not completely absent, adhesion as well as spreading of platelets were distinctly poor and were followed by weak spontaneous aggregation However aggregation was well observed after the addition of either ADP, epinephrine, norepinephrine, thrombin or collagen

Table III Platelet studies. Macroscopic aggregation of normal and afibrinogenaemic platelets (case 1)

Product added	Concentration	Control sec	Case 1 sec
ADP	10^{-4} M	18 4	34.5
	10^{-4} M	22 7	36 0
	10^{-4} M	24 1	39 4
	5×10^{-7} M	26 0	39 6
Epinephrine	$5 4\times10^{-4}$ M	19 0	40 7
	$5 4\times10^{-4}$ M	19 0	47.3
	$2 7\times10^{-4}$ M	19 4	51 0
	1.3×10^{-4} M	20 0	85 4
Norepinephrine	$5 9\times10^{-4}$ M	19 0	28 4
	$5 9\times10^{-4}$ M	19.3	28 4
	$2 9\times10^{-4}$ M	20 0	31 4
	$1 4\times10^{-4}$ M	21 7	82.5
Thrombin	undiluted	coagulated	17.5
	1/4	15 4	60 0
	1/16	17 0	75 0
	1/64	17 6	120
	1/256	21 6	120
	1/1024	23 7	120
	1/4096	25 4	120
Collagen		19 0	17.2

Table II Platelet metamorphosis

	Stages	Control	Case 1
A Recalcified thrombinized PRP	1 Early platelet alterations (ring or bubble forms, etc)	present	present
	2. Aggregates	large	small
	3 Contraction of aggregates to amorphous amorphous form	present	present
	4 Dissolution	present	absent, disaggregation
B Collagen + PRP		as above	as in control

Table V Reaction of afibrinogenaemic platelets (case I) to kaolin

Low-spun plasma + high spun plasma		Kaolin time sec
Patient	Patient	not clottable
Patient	Normal	37.5
Patient	Patient + fibrinogen (final conc. 300 mg%)	39.5
Normal	Normal	31.0

Table VI Reaction of normal platelets suspended in afibrinogenaemic plasma to kaolin

Washed normal platelets suspended in	Kaolin time sec
Normal high spun plasma	33.5
Patients high spun plasma	48.0

Table *III* shows that the time of macroscopic aggregation of the platelets of our patients is longer than that of control at different concentrations of ADP, epinephrine, norepinephrine, thrombin and collagen.

Platelet metamorphosis. Table IV shows that the platelets in the thrombinized or recalcified plasma did undergo early platelet alterations, i.e. formation of ring or bubble forms, but aggregates were smaller as compared to control observations. Contraction of the aggregates occurred and amorphous appearance was assumed. However, a striking feature was the absence of dissolution in the next stage; on the contrary, a disaggregation ensued. Platelet metamorphosis in the presence of collagen suspension occurred normally.

Reaction of afibrinogenaemic platelets to kaolin is shown in table V. Only a mild prolongation of kaolin time was observed when the patients' low spun plasma was mixed with normal high-spun plasma. However, a more distinct abnormal behaviour of normal platelets to kaolin was noted when they were suspended in afibrinogenaemic plasma (table VI).

Discussion

Congenital afibrinogenaemia presents several facets of unusual interest. One remarkable feature is the relative mildness of bleeding tendency in spite of total lack of fibrinogen. Most of the patients have less trouble with bleeding than a haemophiliac. Both the sisters reported here bleed only after injuries; they never had epistaxis, haematuria or haemarthrosis. Even the mild menorrhagia seen in the elder sister is not common. The patients of PINNIGER and PRUNTY [28] and LAWSON [22] had fairly normal menstruation. However, the bleeding tendency is notable soon after birth. Both of our patients had umbilical bleeding, a feature which appears to be fairly common as

QUICK [30] has quoted 12 references on its occurrence The bleeding appears to be more severe during infancy and afterwards the prognosis regarding haemorrhage is quite favourable

An interesting feature is the occurrence of pregnancy in the elder sister which, unfortunately, terminated at 3 months The post abortal bleeding, though prolonged was controlled without any blood trans fusion or hospitalization Presumably, this is the first recorded instance of conception in a patient with congenital afibrinogenaemia

Another danger speculated in these patients is that of a compromise with defence mechanism against infection since fibrinogen is supposed to play a role in body defences The patient of PRENTICE [29] died of fulminating military tuberculosis CASTEX *et al* [8] were able to control a primary tuberculous infection by plasma transfusions However, in both of our patients we failed to find any increased susceptibility to infection

The hereditary nature of the disease was noted even in early studies Consanguinity of the paternal grandparents was recorded in the first patient described by RABE and SALOMON [31] Parents were first cousins in cases reported by MACFARLANE [23], HENDERSON [17], PINNIGER and PRENTICE [28] and FRICK and MCQUARRIE [12] while in other reports grandparents were first cousins [2, 32]

The presence of consanguinity in our cases is also striking but its significance is reduced by the fact that the patients' paternal grand father who is a direct descendent from the consanguinous marriage has a fibrinogen level of 276 mg% and it is only in his wife that a level below 200 mg% is present The mode of inheritance appears to be autosomal recessive In heterozygous states only slight lowering of fibrinogen is observed but homozygous pattern may result in extreme deficiency

The role of plasma fibrinogen in the normal platelet aggregation has been pointed out by several workers [4, 7, 9, 10, 14, 18, 25, 26] INGERMAN *et al* [18] demonstrated that the anomaly of aggregation is more evident spontaneously, in the presence of epinephrine, nor epinephrine, or a small amount of ADP than with the larger amounts of ADP in the presence of collagen or during coagulation

In this investigation afibrinogenaemic platelets showed poor adhesion to glass and weak spontaneous aggregation in their own citrated plasma After the addition of ADP, adrenaline noradrenaline and thrombin aggregation ensued but after a delayed interval

INGEMAN *et al.* [18] reported agreeable findings regarding ADP and thrombin but they noticed no aggregation at all with epinephrine and norepinephrine in comparable concentrations.

Early morphologic metamorphosis of platelets occurred normally in our patient. This finding supports the earlier view that intrinsic thrombin can act on platelets and induce the release of nucleotides [6] and 5-hydroxytryptamine [16], and may act on platelets through another protein than fibrinogen [13, 18, 27].

Normal platelets when suspended in afibrinogenaemic plasma showed delayed reaction to kaolin. INGEMAN *et al.* [18] also quoted a personal communication from HARDISTY to the similar effect and posed the question of whether it was related to platelet phagocytosis.

A new intriguing aspect of fibrinopenia has been brought out by the seemingly paradoxical occurrence of venous thrombosis followed by pulmonary embolism [19]. According to those authors, only platelets and no fibrin could be found in any clot. It is possible that in each patient, the platelets may have carried some absorbed fibrinogen which may have been initially helpful in the mechanical formation of platelet thrombi.

Summary

Congenital deficiency of fibrinogen is described in two sisters aged 7 and 20 years respectively. Both had markedly prolonged umbilical bleeding and easy bruising afterwards. The elder sister has mild menorrhagia and had prolonged post-abortal uterine bleeding after 3 months of gestation. The haematological studies including the platelet behaviour are reported and discussed. The family study indicates the mode of inheritance to be probably autosomal recessive.

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L. P. Holländer und M. Matthes: **Ergebnisse der Bluttransfusionsforschung.** Vol. 10. Bibliotheca Haematologica, No. 32. Bericht der Deutschen Gesellschaft für Bluttransfusion, Graz, Mai 1968. Karger, Basel/New York 1969. 318 p., 92 fig., 72 tab. Preis DM/sFr. 79.–/US $ 18.95/158s.

Die Hauptthemen dieses Kongressberichtes umfassen aktuelle Gebiete der Hämatologie: genetisch bedingte Serumgruppen, neuere Aspekte der Therapie und Prophylaxe des Morbus haemolyticus neonatorum, Automation und Dokumentation im Bluttransfusionsdienst, erworbene Störungen der Hämostase (Verbrauchskoagulopathien). Entsprechend der Themenwahl ist der Nutzerfolg unterschiedlich. In klar begrenzten Gebieten erhält der Nicht-Spezialist in wenigen Seiten eine ausgezeichnete Orientierung, während bei komplexeren und wissenschaftlich noch stark kontroversen Fragen, wie den Verbrauchskoagulopathien, auf Grund von Einzeldarstellungen kein einheitliches Bild zustande kommen kann. Dem hämatologisch interessierten Arzt kann diese Zusammenstellung mehrerer hämatologisch interessanter Themen empfohlen werden. W. H. Hitzig, *Zürich*

G. Duhamel: **Histopathologie du ganglion lymphatique.** Masson, Paris 1969, 186 p., 160 fig. 68 F.

Das Buch verfolgt vorwiegend praktische Zwecke: jede besprochene Krankheit, bei welcher Lymphknotenveränderungen auftreten können, wird zunächst vom klinischen Standpunkt kurz geschildert, es folgt jeweils eine Anzahl Mikrophotographien mit einer ausführlichen histologischen Beschreibung der Befunde, einem Kommentar zur Differentialdiagnose und einer Erläuterung der möglichen Fehldiagnosen. Die eingehende klinische und histopathologische Besprechung von 12 ausgesuchten Beobachtungen, mit den entsprechenden Diskussionen zur Differentialdiagnose, bildet eine sehr interessante und vor allem instruktive Vervollständigung des Werkes. Als Schlusskapitel wird eine Zusammenstellung der speziellen technischen Angaben gebracht, die bei Fixierung, Einbettung und Färbung von Lymphknoten-Biopsien berücksichtigt werden sollten.

Im grossen und ganzen ist die vorliegende Zusammenstellung sehr nützlich, sie wird sowohl dem Kliniker wie dem Histopathologen willkommene Hinweise vermitteln, auch wenn leider die Bilder in Schwarzweiss-Reproduktion nicht immer sehr gelungen sind, wie es eigentlich in einem Atlas sein sollte (vor allem nicht die Übersichtsaufnahmen!). Die Literatur berücksichtigt eingehend das französische und anglo-amerikanische Schrifttum, nicht aber die Literatur deutscher Sprache. F. C. Rollet, *Basel*

Acta haemat. 43 129-143 (1970)

A Study of the Effects of Possible Toxic Metabolites of Uraemia on Red Cell Metabolism

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Poor haemopoiesis as a result of low production of erythropoietin is one factor in the anaemia of uraemia [5] but there is in some patients a short red cell survival [19]. Normal red cells also have impaired survival when transfused into uraemic subjects [30] and this suggests that serum factors contribute to the anaemia. Moreover erythrocytes from normal subjects show increased autohaemolysis and potassium loss when incubated in serum from uraemic patients [14].

In this report a study has been made of their effects on erythrocytes of those metabolites that may contribute to the toxaemia of uraemia. Such metabolites include phenols [10, 20, 29, 32] and phenolic acids [16, 21] derived from bacterial degradation in the gut or by endogenous metabolism of phenylalanine, tyrosine and tryptophan, aliphatic amines [33], oxalic acid [11], and guanidines including the recently discovered metabolite guanidino-succinic acid [7].

Such metabolites may induce premature red cell ageing. The present study reports the effects of suspected toxic substances on red cell shape, on osmotic fragility and autohaemolysis, on the formation of methaemoglobin and on the uptake of radio-active phosphorus and potassium. Measurements have also been made of their action on the enzymes pyruvate kinase and glutathione reductase, these being two key points in the metabolism of the cell, and on the reduced glutathione content of cells during incubation in buffered media and of red cells taken from uraemic patients before and after in vivo dialysis.

Methods

The substances given in table I were selected for study and made up in 0.9% saline. Table I gives the final concentration used in the incubation studies, they are well above the values obtained in uraemia.

Incubation was carried out in GEY's solution [13] which was buffered with an equal volume of 0.01 M phosphate buffer to the appropriate pH (pH 6.8, 7.4, 7.8). Red cells were drawn from a normal donor into heparin, centrifuged and the buffy layer removed, then washed twice in 0.9% NaCl before use. 1,000 units of sodium heparin (Weddell Pharmaceuticals, London) which contains 0.15% chlorocresol was used for 50 ml of venous blood. The packed cell volumes of these suspensions were measured at the commencement of the incubation time, by spinning at 2,000 *g* for 30 minutes. Two volumes of washed packed red cells were added to one volume of the buffered GEY's solution plus one volume of the reagent in 0.9% NaCl and incubation was carried out at 37°C for the stated time period with continuous shaking of the tubes.

Reduced glutathione (GSH) estimations were made, in triplicate, according to the method of BEUTLER *et al.* [2] in which the yellow colour produced by DTNB (5.5-dithio-*bis*-2-nitrobenzoic acid) was measured at 412 nm. The glutathione content of the red cells is obtained from the whole blood GSH by correction for the packed cell volume.

Pyruvate kinase was estimated by the method of TANAKA *et al.* [36]. The unit is that activity resulting in the conversion of one micromole NADH to NAD per minute by 10^{10} erythrocytes. The change during the 4th to 14th minute is used for calculation purposes as this is linear.

For the estimation, haemolysates were prepared after two hours' incubation of red cell suspensions at 37°C in the various reagents in GEY's solution buffered at pH 7.4. The haemolysates were diluted in reagent to give a haemoglobin of 0.15 g% for the estimation. This gives a value for red cell pyruvate kinase that has been exposed within the red cell for two hours at 37°C to the appropriate reagent and which is then suspended as a haemolysate in that reagent for the test.

Glutathione reductase. Red cells were incubated in the presence of the various reagents for two hours at 37°C in GEY's solution buffered at 7.4, and the red cell haemolysate was then prepared. Glutathione reductase was measured according to the method of WEST *et al.* [42] in which 0.2 ml haemolysate containing glutathione reductase, 1.5 ml of 0.01 M phosphate buffer (pH 7.4), 0.5ml reagent in GEY's solution buffered at pH 7.4 and 0.1 ml of 0.002 M NADPH solution were incubated together at 37°C for 20 min. Oxidised glutathione (0.1 ml of 0.033 M) was added to the mixture and the change of optical density at 340 nm measured at 30 second intervals for 6 min. A blank was used for each reagent without NADPH. A unit is a change of optical density of 0.001 per ml of enzyme per minute and is equivalent to 0.48×10^{3} μm of NADPH oxidised per ml of enzyme per minute. Control values were 50 units per minute per ml packed red cells.

^{32}P uptake. Radioactive phosphorus (1 μc ^{32}P as orthophosphate) was added to the incubation mixture in GEY's solution buffered at pH 7.4 and incubation was carried out at 37°C with frequent shaking, after which time the cells were washed three times with 0.9% NaCl. After the final wash the cells were lysed and made up to 10 ml with water for counting in a scintillation counter.

Methaemoglobin was estimated by the method of EVELYN und MALLOY [12]. The incubation period was 2 hours at 37°C.

Autohaemolysis was estimated by the method of DACIE and LEWIS [9] after incubation for 48 hours at 37°C.

Heinz body formation. Cells were stained by adding one volume of blood to 4 volumes 0.5% methyl violet in saline. After 10 min one drop of mixture was smeared on a slide and examined immediately.

^{42}K uptake was performed by the method of SOLOMON [34] by measuring the disappearance of ^{42}K from 0.1 ml supernatant samples, taken after 2 and 4 hours' incubation of red cells in reagent in GEY's solution buffered at pH 7.4. From the initial and final serum and whole blood potassium concentration, measured by flame photometer, the uptake of total potassium was also calculated. The results are expressed as the average potassium influx in mEq/l of cells per hour and as the total uptake of radio-potassium at 2 and 4 hours.

Results

GSH. The effects on the red cell GSH of incubation of normal cells for 2 hours and overnight at pH 6.8, for 2 hours and overnight at pH 7.4, and for 2 hours at pH 7.8, was ascertained. The results are given in table I and are expressed as standard deviations. It will be seen that the most significant deviations are given on incubation of normal cells for 2 hours at pH 7.4 and that guanidine, oxalate, mandelic acid, ammonia and phenol depress the GSH values, whereas these are raised by the amines dimethylamine and ethanolamine and by indoleacetic acid. The metabolites of tryptophan lower the GSH values as do creatine, creatinine and uric acid. Cysteine gave high values because its own free-SH groups give a yellow colour with DTNB. Experiments were also carried out in which red cells were incubated for 4 and 6-hour periods at pH values of 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8, to determine the effect upon red cell GSH. Only at pH 6.8 was there any significant effect (lower than 3 SD) on the reduced glutathione.

To make some comparison with the situation in chronic uraemia, the glutathione instability of uraemic red cells incubated in their own plasma for 2 hours, with and without phenylhydrazine, was made. The results (as GSH in mg per 100 ml packed cells) of 12 uraemic values and 12 controls are shown in table II. It can be seen that there is no significant difference between the two groups but uraemic cells show a slight lowering of GSH with phenylhydrazine.

In addition, some GSH levels were measured on chronic uraemic patients before and immediately after *in vivo* dialysis (table III). There was a small overall reduction of GSH shown by a mean value of 0.8 mg GSH per g of haemoglobin (standard deviation ±2.1). Wide fluctuations in the absolute glutathione content of the red cells and in the mean corpuscular haemoglobin concentration (MCHC) are evident with dialysis. Values in mg/100 ml red cells are given to enable comparison with other authors. The variations in MCHC as a result of

Table I Results of reduced glutathione estimations on red cell incubates

Reagent	Incubation reagent strength mg/100 ml	pH 6 8 2 h	Over-night	pH 7 4 2 h	Over-night	pH 7 8 2 h
Urea	250	–	–	–	–	+1
Creatine	15	–1	–	–2	–	+1
Creatinine	15	–1	–	–1	–	+1
Uric acid	8	–	–	–2	–	+1
Guanidine	50	–	–1	–3	–	–
Oxalate	50	–1	–1	–3	–1	–
Ammonia	4	–	–	–1	+1	–
Mandelic acid[1]	50	–	–2	–3	–1	+1
Phenol	25	–1	–1	–3	–	+1
Indole-3-acetic acid	250	–	+1	+1	+1	+1
Dimethylamine	1,600	+1	–	+1	–	+3
Ethanolamine	500	+1	–	+3	–	+1
Cysteine	5	+5	+5	+5	+5	+3
Glycine	5	+1	+3	+3	–	–
Hippuric acid	5	–	–	–	–	–
Glycollic acid	5	+1	+3	+3	–	–
Sodium benzoate	1	–	+2	–	–	–
Magnesium sulphate	25	+1	–	+3	–	–
Kynurenine	0.200	–	–	–1	–	–
Anthranilic acid	0 250	+1	–	–1	–	–
Cinnamic acid	0.500	–	–	–3	–	+1
3-Hydroxy-anthranilic acid	0.250	–	–	–2	–	–
Nicotinamide	0 400	–	–	–3	–	–
Control values (mg/100 ml red cells)		55 0 ±8 4	55 0 ±6 7	66 0 ±3 1	55 0 ±8 0	60 ±10.5

The reduced glutathione values at zero time were 66 0±3 1 mg/100 ml red cells Values in table are expressed as standard deviations.

[1] Hydroxy phenyl-acetic acid

dialysis reflect changes in cellular hydration. When correction is made by expressing the glutathione content of the cells per g of haemoglobin there is no consistent change and for the series as a whole no significant change. A single dialysis of a chronic uraemic patient does not render that patient non-uraemic but there may be a threefold reduction of blood urea (and probably of other metabolites).

Table II

Sample	Before incubation	After incubation no additive	With phenylhydrazine
Normal	65.6 ± 9.2	58.2 ± 6.5	50.9 ± 9.25
Uraemic	65.6 ± 10.4	55.9 ± 4.45	46.4 ± 9.05

Table III Changes in red cell GSH after dialysis of 15 uraemic patients

mg/100 ml red cells		*mg g Hb*		MCHC	
Before dialysis	After dialysis	Before dialysis	After dialysis	Before dialysis	After dialysis
98.0	44.0	9.4	3.35	36.0	33.0
104.0	123.0	13.7	16.2	36.0	40.0
191.0	74.0	15.2	8.4	35.0	32.6
50.5	57.0	5.0	6.95	42.0	34.2
57.0	63.0	6.7	7.9	28.4	34.2
54.0	55.0	6.1	6.7	30.7	33.0
65.5	70.5	8.85	8.0	33.6	31.0
70.0	64.0	7.3	6.3	32.0	32.6
45.0	44.0	6.25	5.7	32.8	33.0
40.0	40.0	6.7	7.3	33.0	32.0
82.0	84.5	12.6	12.1	29.0	29.0
79.0	56.0	10.1	7.2	27.0	27.0
42.0	34.0	4.18	3.46	33.0	30.0
68.5	53.5	9.0	6.2	20.0	23.0
64.5	72.0	8.3	9.2	27.0	27.0

It is concluded that there is no significant reduction of GSH of the red cell in chronic uraemia, that dialysis of patients does not alter significantly the GSH of the red cells, that a pH sufficient to cause marked instability is unusual in life, but that since some of the agents that are retained in uraemia lower the glutathione to a certain extent and others raise the value, a state of balance may actually exist. Clearly this might vary from patient to patient. An accurate assessment will only be forthcoming when there are sensitive techniques for estimating blood levels of the possible toxic substances.

^{32}P uptake Triplicate uptake measurements of ^{32}P into normal red cells from Grey's solution buffered to pH 7.4 and to which the appro-

Table IV. Uptake of ^{32}P orthophosphate into red cells incubated with various substanc

Reagent	2 h	SD[1]	4 h	SD[1]
Control Mean of 6 values	4,478 ±280		6,563 ±180	
Urea	4,677	–	7,053	+2
Creatine	5,492	+3	7,448	+4
Creatinine	4,977	+1	6,863	+1
Uric acid	5,190	+2	7,200	+3
Guanidine	4,736	–	6,573	–
Oxalate	5,634	+3	8,454	> +4
Ammonia	4,738	–	7,628	> +4
Mandelic acid	5,193	+2	7,643	> +4
Phenol	3,994	–1	6,293	–1
Indole-3-acetic acid	5,166	+2	7,118	+3
Dimethylamine	4,871	+1	7,299	+4
Ethanolamine	4,144	–1	6,707	–
Cysteine	5,563	+3	6,453	–
Glycine	5,281	+2	7,952	> +4
Hippuric acid	4,746	–	6,381	–1
Glycollic acid	4,426	–	7,485	> +4
Sodium benzoate	4,483	–	7,123	+3
Magnesium sulphate	4,863	+1	6,782	+1
Kynurenine	5,346	+3	7,124	+3
Anthranilic acid	4,662	–	6,961	+2
Cinnamic acid	4,508	–	6,952	+2
3-Hydroxy-anthranilic acid	4,623	–	7,356	+4
Nicotinamide	4,726	–	6,802	+1
Hydroxylamine	5,673	+4	7,783	> +4
Thiocyanate	5,018	+1	7,167	+3

Reagent	mg%	2 h	SD	4 h	SD
Oxalate	50.0	5,602	> +4	9,173	> +4
	25.0	5,431	+3	9,683	> +4
	10.0	5,365	+3	8,704	> +4
	2.5	4,875	+1	7,382	+4
Ammonia	4.0	4,932	+1	8,224	> +4
	2.0	4,918	+1	8,183	> +4
	1.0	4,875	+1	7,624	> +4
	0.5	4,892	+1	7,381	+4
Mandelic acid	50.0	5,054	+2	7,963	> +4
	25.0	4,609	–	6,873	+1
	6.5	4,513	–	6,894	+1
	2.5	4,571	–	6,800	+1

Table II (continued)

Reagent	mg%	2 h	SD	4 h	SD
Phenol	25 0	4 643	–	6,754	+1
	12.5	4,581	–	6,537	–
	5 0	4 624	–	6,504	–
	1.25	4,378	–	6 089	–2

Counts in 300 sec are recorded
[1] The standard deviation is taken from the mean of 6 control values.

priate reagent had been added, were made after 2 and 4 hours' incubation The values as counts in 300 sec, are recorded in table IV where it may be seen that most of the reagents used, with the notable exception of phenol, caused an increased uptake of radioactivity compared with the control values

[illegible]

100 ml are sufficient to increase phosphorus uptake Phenol, gives values for ^{32}P uptake similar to control values but low concentrations depress ^{32}P uptake The results are given as standard deviations from the mean of 6 control values, that is $\pm$ 280 (counts in 300 sec) for the 2 hour values and $\pm$180 for the 4-hour uptakes Similar measurements were made with red cells suspended in GEY's solution buffered at different pH values, and it was found that progressive reduction of pH below 7 4 was associated with progressive impairment of ^{32}P uptake

In further experiments, measurements of ^{32}P uptake were made on red cells taken from uraemic and normal persons and suspended for up to 6 hours in GEY's solution at pH 7 4 The mean of 6 values (counts per 300 sec) for each group, with a correction for PCV are given in table V The *initial depression of uptake in the uraemic red cells* is quite striking The results suggest that by the sixth hour a toxic metabolite has been washed out of the uraemic cells or that a possible intracellular acidosis has been *corrected In view of the fact that no* nitrogenous product depressed ^{32}P uptake but that an acid pH did, the depression of uptake by the uraemic cells may be due to a lowering

Table I

Sample	Uptake of ^{32}P in counts		
	2 h	4 h	6 h
Uraemic	1 972 ± 453	3 949 ± 687	5 146 ± 790
Normal	3 223 ± 180	4 910 ± 139	5 862 ± 121

of their intracellular pH. When the cells were first removed from chronic uraemic patients the intracellular pH may have been low but later restored to normal in the buffer at pH 7.4.

Pyruvate kinase and glutathione reductase (table VI). The results of estimation of pyruvate kinase show that all the reagents tested impair the enzyme activity. Guanidine is particularly potent, but even the usually recognised metabolites of uraemia (urea, creatinine and uric acid) have quite a marked effect on this enzyme. In the case of glutathione reductase, it is of interest that some metabolites such as glycine and hippuric acid, indole acetic acid and urea, impair the action whereas others, such as oxalate, ammonia and mandelic acid and phenol, enhance the activity.

Methaemoglobin formation. As seen in table VI some of the various reagents were able to produce methaemoglobin in amounts that are known to exist in uraemia.

Autohaemolysis. None of the reagents used produced decisively abnormal values.

Osmotic fragility. The red cells of the peripheral blood in uraemia show no change in osmotic fragility. Fragility studies were performed after 2 hours incubation of normal red cells in the appropriate reagents (mandelic acid, oxalate, phenol, guanidine urea, ammonia and indole acetic acid) and none of these substances showed any direct *in vitro* effect upon the fragility of the red cells.

However, a shift of pH below 7.2, begins to increase red cell fragility which probably parallels impaired red cell glycolysis (or phosphorus uptake). Detailed studies of the effect of pH on red cell fragility were reported by PARPART *et al.* [28].

Red cell shape. Red cells were incubated in GEY's solution buffered at pH 7.4 and with the various reagents for up to 24 hours. In the case of cells in buffered GEY's solution it was found that abnormal shapes began to appear after about 15 hours incubation. Many of the reagents

Table 11 The effect of various substances on methaemoglobin formation. Pyruvate kinase and glutathione reductase activities

Reagent	Methaemoglobin % Hb	Pyruvate kinase units 10[10] RBC	Glutathione reductase units/min/ml RBC
Controls	0 6	3.3–4.0	50
Urea	0 6	1.9	37
Creatine	0	2 11	35
Creatinine	0 6	1.39	52
Uric acid	0.3	1.22	46
Guanidine	1.2	0.55	52
Oxalate	1.5	1 43	90
Ammonia	0 6	2.32	93
Mandelic acid	1.8	1 44	62
Phenol	2.4	1 68	70
Indole-3-acetic acid	1.5	1 75	24
Dimethylamine	0.3	2.26	56
Ethanolamine	0 6	3 0	40
Cysteine	0	–	135
Glycine	0	–	53
Hippuric acid	0	–	6
Glycollic acid	1.2	–	5
Sodium benzoate	3 0	–	68
Magnesium sulphate	3 0	–	68
Kynurenine	3 3	1 47	56
Anthranilic acid	0.9	2.24	62
Cinnamic acid	0	1.06	43
3-Hydroxy-anthranilic acid	3 6	3 0	21
Nicotinamide	0	1.36	28
Hydroxylamine	3 0	–	0
Thiocyanate	1.2	–	72

used induced the formation of 'formes en anneau' (1) (urea, guanidine, phenol, ammonia, mandelamine, indole and oxalate) together with abnormally crenated and distorted cells after only two hours' incubation Further, a shift of pH below 7 2 would effect a similar change in a very short time

Heinz body formation No Heinz bodies were found in the blood of uraemic patients or in incubated red cells

[42]K uptake and potassium efflux studies The results are given in table VII The control values for the influx of potassium in mEq/l of cells

Table III Uptake by red cells of ^{42}K at 2 and 4 h incubation time

	^{42}K uptake counts in 300 sec 2 h	4 h	2–4th h	Average K uptake from medium mEq/l cells per hour
Controls	2,000	5,580	3,580	1.47
	2,200	6,100	3,900	1.47
	2,550	6,850	4,300	1.47
Urea	4,100	7,750	3,650	1.50
Creatine	2,600	6,650	4,050	2.18
Creatinine	3,300	9,600	6,300	2.18
Uric acid	3,100	6,650	3,550	1.91
Guanidine	2,000	8,000	6,000	2.09
Oxalate	4,200	6,930	2,780	1.40
Ammonia	3,250	7,750	4,500	1.73
Mandelic acid	1,350	8,050	6,700	2.36
Phenol	2,550	8,800	6,250	2.27
Indole-3-acetic acid	1,550	8,870	7,370	2.45
Dimethylamine	1,170	6,820	5,750	2.45
Ethanolamine	1,750	8,070	6,320	2.27
Cysteine	2,000	8,350	6,350	2.18
Anthranilic acid	2,000	9,000	7,000	2.27
3-Hydroxy anthranilic acid	2,000	9,400	7,400	2.27

The uptake of ^{42}K between the second and fourth hour correlates with the average potassium uptake per hour. Reagents are separable into two groups according to whether the uptake is greater than 5,000 counts (in 300 sec) between the second and fourth hour. Urea and oxalate are anomalous as the uptake was greatest in the first two hours and thereafter K influx declined.

per hour approximate to the values obtained by SOLOMON [34]. It will be seen that all the reagents studied produce an increase of potassium influx and of radio potassium uptake especially between the second and fourth hour and that this value correlates with the total potassium uptake per hour. However, urea and oxalate are anomalous as the uptake was greatest in the first 2 hours and then fell off and the average potassium uptake (mEq/l cells/hour) was not then different from the control values. The reagents are in fact separable into two groups according to whether the uptake is greater than 5,000 counts between two and four hours. The substances which caused an uptake of over 8,000 counts in 4 hours and which also produced a potassium

influx greater than 2 0 mEq/l of cells per hour were phenol and mandelic acid, creatinine and guanidine, anthranilic and three hydroxyanthranilic acids, indole acetic acid, ethanolamine and cysteine There was no direct correlation with those substances that produced the greatest effect on ^{32}P uptake

Discussion

The known facts concerning altered red cell metabolism in uraemia are conflicting probably because in some patients there is a higher percentage of young cells with higher enzyme values Some authors have reported that glucose uptake by the cells is increased [23] or decreased [24, 25] there is increased methaemoglobinisation [3] and *reduced glutathione can be increased [43] or normal [41] in chronic* uraemia but low in acute anuria [37] Also it is known that the enzyme hexokinase is very sensitive to acidosis [3] there is reduced activity of erythrocyte pyruvate kinase [3] and unimpaired activity of glucose-6-phosphate dehydrogenase but increased activity of glutathione reductase [6] In addition there may be failure of the sodium pump [8, 24, 39] but a high ATP [22 23] and increased efflux of potassium and uptake of ^{32}P is low [22, 23]

Table VIII gives a synopsis of some of the data and it will be seen that phenol and the phenolic acid, mandelic acid, together with acidosis could effect most of these changes Guanidine is also a very toxic metabolite and lowers the glutathione content of the cell and the pyruvate kinase activity and actually increases the potassium influx Urea, creatinine and uric acid produce minor changes but clearly have no great toxicity Oxalate and ammonia produce some changes akin to phenol and indole acetic acid the amines and the tryptophan metabolites show variable toxic effects but no consistent pattern

It is clear that a single toxic metabolite will not account for the demonstrable abnormalities but certain classes of metabolite can be defined The overall action is complex thus several of the reagents have an [illegible]
on glutat [illegible]
synthesis [illegible]
caused an increased uptake of ^{32}P, an action opposite to that occurring in uraemia but nevertheless indicating a threat to the integrity of the

Table VIII Synopsis of conclusions Normal red cells treated with various substances

Uraemia		Urea	Mandelic acid	Phenol	Indole/amines	Acidosis	Guanic
Glutathione	low (?)	+	++	++	0	++	++
G reductase	raised	0	+	+	0		0
Methaemoglobin	raised	0	++	+++	+/0		++
Pyruvate kinase	low	+	+	+	+/0	+	+++
^{32}P uptake	low	0	0	+	0	++	0
^{42}K uptake	increased (?)	0	++	++	++		++
Cell crenation		+	++	++	++	fragile	++

0 = no effect of added reagent + = positive effect of the reagent.

cell In hereditary spherocytosis [17] increased uptake of phosphorus and increased glycolysis occur as part *of an attempt to increase sodium* pumping and similarly when normal cells are exposed to sulfhydryl inhibitors [18], or to sodium fluoride [15] The increased uptake of ^{42}K induced by many of the reagents is difficult to explain but may be due to the breakdown of larger to smaller molecules within the cell, especially associated with the intracellular production of phosphates and so there might be an indirect link with reduced phosphorus uptake ATP is always present within the erythrocyte when the intracellular potassium is rising [44] and when ATP falls potassium leaks into the medium An early uptake of potassium with a dramatic fall off has been shown with urea at 250 mg% and with oxalate In fact the rate of glycolysis is partly controlled by the magnitude of ion transport, the link being that ADP produced by ATPase stimulates 3 phosphoglycerate kinase [15]

It has been the hope of many investigators to explain the 'burr cell If by this we mean the fragmented cell, then this occurs in association with intravascular coagulation [4], a process involved in the pathogenesis of some types of renal disease [38] and is indeed an indication of short red cell survival [31] On the other hand crenated cells can be produced by washing to remove cholesterol [26] and spicule formation is a feature of pyruvate kinase deficiency [27] Table VIII shows that guanidine is the most potent inhibitor of pyruvate kinase but phenol and urea are depressors

The results here indicate that a combination of phenolic compounds together with acidosis is capable of producing crippling metabolic derangement of the red cell but that guanidine and its derivatives may have similar effects.

Summary

A study has been made of possible toxic metabolites of uraemia on red cell shape, autohaemolysis and fragility, on reduced glutathione and methaemoglobin content of red cells, on glutathione reductase and pyruvate kinase activity and on ^{32}P and ^{42}K uptake by red cells. Analysis of the effect of individual compounds gives some insight into the complex metabolic situation that must exist in uraemia. Most retained compounds are detoxicated but the combined effects of phenolic acids and guanidine compounds with the acidosis of uraemia would appear to be sufficient to account for shortening of the red cell life span.

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Die makrozytäre Regeneration der Erythropoese

Tierexperimentelle Untersuchungen bei der Phenylhydrazinhämolyse[1]

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Die Phenylhydrazinhämolyse ist zum Studium der Regeneration der Erythropoese in besonderem Masse geeignet. Denn durch das Gift werden alle zirkulierenden Erythrozyten zerstört, die plötzlich einsetzende schwere Anämie löst eine starke Stimulierung der Erythropoese aus, und die neugebildeten Zellen können dann ohne Untermischung mit der vorbestehenden Population untersucht werden.

Wie aus zahlreichen Untersuchungen bekannt ist, resultiert eine erhebliche makrozytäre Regeneration. Die neugebildeten Erythrozyten sind aber z. T. nach wenigen Tagen nicht mehr nachweisbar. Sie hämolysieren und/oder schrumpfen, wie wir in den vorliegenden Untersuchungen nachgewiesen haben. Aus diesen Befunden bei maximaler Stimulierung der Erythropoese dürfen aber keine Rückschlüsse auf die Erythropoese im normalen Fliessgleichgewicht, wie z. B. auf die Grösse der Retikulozyten [101] gezogen werden. Dieser ist vielmehr die «physiologische» Stimulierung der Erythropoese vergleichbar, die wir am Modell der «Verdünnungsanämie» beschrieben haben. Hier kommt es zu einer Mehrproduktion von normal grossen Erythrozyten mit normaler Lebenszeit [6, 9].

Methoden

[illegible] 2,5–5,0 kg Trockenkost (Hope farms Standard [illegible] Vorbehandlung [illegible]

[1] Mit Unterstützung der Deutschen Forschungsgemeinschaft

Acetylphenylhydrazin (APH): 0,5% Lösung frisch hergestellt, Einzeldosis (soweit nicht anders angegeben) 15 mg/kg, meist wurde an 3 aufeinanderfolgenden Tagen je eine Dosis s.c. injiziert, die Tage nach APH wurden vom 1. Injektionstag an gezählt.

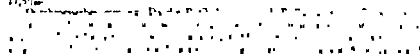
Bei 18 Tieren wurden auf dem Höhepunkt der Retikulozytose 100 µg/kg Actinomycin D (Dactinomycin)* i.v. injiziert. Da viele Tiere frühzeitig während des Versuchs starben, konnten die Ergebnisse dieser Versuchsreihe nur von 6 Tieren ausgewertet werden. Fünf Tieren wurde nach 3 Tagen die gleiche Dosis Actinomycin D nochmals gegeben.



12000 g, Doppelbestimmung.

Aus diesen Messwerten wurden Hämoglobingehalt (Hb_E = MCH), Hämoglobinkonzentration (MCHC) und mittleres Erythrozytenvolumen (MCV) berechnet.

Retikulozyten: Färbung mit Brillantkresylblau 1% in 0,9% NaCl (3 Tropfen Blut + 3 Tropfen Farbstoff) 30 min in verschlossenem Röhrchen, Ausstriche. Die Farbstofflösung ist hypertonisch. Vergleichende Grössenmessungen (Price-Jones-Kurven) mit einer isotonischen Brillantkresylblaulösung (in 0,7% NaCl, 300 mOsm) ergaben jedoch keine Unterschiede. Die Abnahme des Zelldurchmessers bei Vitalfärbung (im Unterschied zur panoptischen Färbung) ist durch den Farbstoff bedingt [105]. Auszählung von je 1000 Erythrozyten, Angabe in ‰ Retikulozyten bzw. Umrechnung auf Retik./mm³. Die Retikulozytenzahlen liegen bei dieser Methode etwas höher als bei Giemsa-gegengefärbten oder Feuchtpräparaten. Normalwerte (Mittelwert von 50 unbehandelten Kaninchen): 37,5 ± 17,5‰.



und Genauigkeit ergibt.

Innenkörper: Färbung und Auszählung wie bei den Retikulozyten. Dabei erscheinen die Innenkörper hellblau, die Substantia granulo-filamentosa blauviolett.

Untersuchung einzelner Schichten des Erythrozytensedimentes: Heparinblut wurde in eigens hierfür hergestellten schmalen Glasröhrchen 20 min zentrifugiert (1000 g). Nach Abheben des Plasmaüberstandes und der Leukozytenschicht wurde das Erythrozytensediment in genau 3 Teile geteilt, das obere und untere Drittel mit etwas Plasma verdünnt und die mittlere Schicht verworfen.

Messung der Volumenverteilungskurve der Erythrozyten: Suspensionsmedium: 390 ml n/15 Na_2HPO_4, 59 ml n/15 KH_2PO_4, 69 g NaCl, 44 ml Formalin 40%, demineralisiertes Wasser ad 10000, pH 7,4–7,55, 293–295 mOsm. Der Puffer wurde vor Gebrauch gefiltert. Leerwertzählung bei der verwendeten elektrischen Einstellung: 100–200 Partikel ml. Blutverdünnung 1:50000. Messung mit Coulter Counter Modell B mit automatischer Registriereinrichtung (Plotter J). Kapillaröffnung 100 µ. Messung mit maximaler Verstärkung (1/AMP = 1/8), um den Messfehler möglichst niedrig halten zu können (1/APC = ½). Unterer Schwellenwert zur Erythrozytenzählung: D 12. Volumeneichung aus dem MCV-Wert einer Blutprobe (Mittelwert aus 10fach-Bestimmung von Hämatokrit, Erythrozytenzahl und Volumenverteilungskurve).

* Wir danken der Firma Sharp & Dohme, München, für die Überlassung der Versuchsmengen Dactinomycin.

Ergebnisse

Hämolyse durch Acetylphenylhydrazin (APH), Stimulierung der Erythropoese, Hemmung durch Actinomycin D

Nach APH kommt es innerhalb weniger Tage zu einer schweren Anämie, die eine starke Mehrproduktion von Erythrozyten auslöst. Die Retikulozyten steigen am 5. bis 6. Tag nach der 1. Injektion auf 500 bis 700‰. Die neugebildeten Zellen sind ausserordentlich gross und enthalten mehr Hämoglobin als normale Erythrozyten; die Volumenzunahme ist aber grosser als die Hämoglobinzunahme, so dass eine Abnahme des MCHC-Wertes resultiert [s. auch 2, 28, 76, 80, 100, 101]. Nach weniger als 2 Wochen sind die Ausgangswerte wieder erreicht.

Alle Erythrozyten, die sich während der Einwirkung des APH in der Zirkulation befinden und Innenkorper bilden, werden innerhalb weniger Tage hämolysiert. Dies wurde durch gleichzeitige ^{51}Cr-Markierung und Auszählung der Innenkorper nachgewiesen: Bei 5 Tieren wurde am Tag der 1. APH-Injektion eine autologe ^{51}Cr-Markierung vorgenommen und in den folgenden Tagen die Aktivität gemessen und der Anteil der Innenkorper (‰) gezählt. Die etwa parallel verlaufende Abnahme von Radioaktivität und Zahl der Innenkorper spricht dafür, dass nach 9–11 Tagen alle vergifteten Erythrozyten aus dem Kreislauf eliminiert sind. Die Zerstorung der gesamten Zellpopulation bildet die Voraussetzung dafür, dass während der anschliessenden Regenerationsphase die neugebildeten Erythrozyten ohne Beimischung älterer Zellen untersucht werden konnen.

In einer zweiten Versuchsserie (6 Kaninchen) wurde auf dem Hohepunkt des Retikulozytenanstiegs die Erythropoese vollständig gehemmt. Dies geschah durch intravenose Injektion von Actinomycin D, dessen Wirkung auf die Erythropoese *in vitro* [26, 35] und *in vivo* [49, 103] eingehend untersucht worden ist.

Actinomycin D kann in seiner Wirkungsweise auf die Erythropoese gewissermassen als Erythropoetin-Antagonist angesehen werden, d.h. es wirkt nur in den ganz frühen Stadien der Erythropoese auf die Zellbildung und beeinflusst nicht die späteren Ausreifungsstadien [33, 49, 53, 78, 79], ist also kein allgemeines Mitosegift.

Die Grossenmessungen der Erythrozyten konnten dadurch an einer recht einheitlichen «Kohorte» vorgenommen werden, unbeeinflusst vom Zustrom weiterer Zellen aus späteren Phasen der Regeneration.

Tabelle 1 Differenzierung der Retikulozyten nach Aktivierung der Erythropoese durch Phenylhydrazinhämolyse (25.–27. 8.) und Hemmung durch Actinomycin D (30. 8.) Auszählung von je 200 Retik. (K 103)

Datum	Ery mm³ × 10⁶	Retikulozyten Gesamt		I		II		III		IV	
		‰	pro mm³ × 10³	‰	pro mm³ × 10³	‰	pro mm³ × 10³	‰	pro mm³ × 10³	‰	pro mm³ × 10³
31. 8., 8 Uhr	1,60	501	803	17	137	48	385	22	177	13	104
31. 8., 17 Uhr	1,51	587	886	9	80	46	408	34	300	11	98
1. 9., 7 Uhr	1,40	415	580	1	6	12	70	53	307	34	197
1. 9., 17 Uhr	1,28	313	440	–	–	2	9	34	150	64	281
2. 9., 7 Uhr	1,23	94	115	–	–	–	–	5	6	95	109
2. 9., 17 Uhr	1,16	23	27	–	–	–	–	–	–	100	27

Der Eintritt der vollständigen Erythropoesehemmung wurde a) durch Retikulozytenzählung und -differenzierung und b) durch Bestimmung der ^{59}Fe-Inkorporationsrate nachgewiesen.

a) Die Retikulozyten fallen 4–5 Tage nach Actinomycin D auf 0 ab. Schon vorher kann der Eintritt der Hemmung durch Differenzierung der Reifestadien [44] nachgewiesen werden (Tab. I): Wird der Retikulozytennachschub plotzlich unterbunden, dann reifen die vorhandenen Retikulozyten aus, d.h. sie verschwinden in der Reihenfolge I–IV, bis schliesslich nur noch ausgereifte Erythrozyten zu finden sind. Dieser Prozess der Ausreifung *in vivo* gleicht somit dem, wie er durch *in-vitro*-Studien [4, 11, 44, 81] bekannt ist. Die aufwendige Retikulozytendifferenzierung wurde bei 2 Tieren durchgeführt. Bei den ubrigen wurde der Nachweis der vollständigen Erythropoesehemmung durch

b) die Messung der ^{59}Fe-Inkorporation erbracht. Bei 4 Tieren wurden am 3. Tag nach der 1. Actinomycin-D-Injektion 10 μCi ^{59}Fe

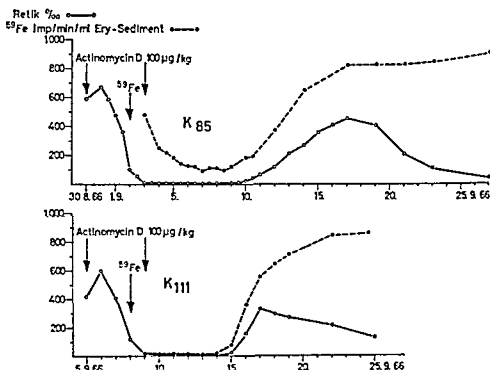


Abb. 1. Wirkung von Actinomycin D auf Retikulozytenzahl und ^{59}Fe-Inkorporation. Oben: Vollblut. Unten: gewaschene Erythrozyten.

i v injiziert In Abständen von 1–3 Tagen wurde Blut entnommen, die Aktivität nach Ende des Versuchs von allen Proben (je 0,5 ml Vollblut) gemessen und in Imp/min/ml Ery-Sediment umgerechnet Die Radioaktivität nimmt nur ganz allmählich ab (Abb 1, obere Hälfte) und ist während des gesamten Stadiums der Erythropoesehemmung im Plasma nachzuweisen, die ^{59}Fe Clearance ist also enorm verlängert (normal bei Kaninchen t/2 = 60–80 min [20]) Mit Wegfall der Actinomycin D Wirkung steigen Retikulozytenzahl und ^{59}Fe Inkorporation parallel an Werden die Blutproben 1 × in 0,9 % NaCl gewaschen und die Aktivitätsmessungen im plasmafreien Erythrozytensediment vorgenommen, dann ist für die Dauer der Actinomycin D Wirkung keine Radioaktivität im Blut nachweisbar (Abb 1, untere Hälfte)

Vom Zeitpunkt des Abfalls der Retikulozytenzahlen bis zu ihrem Wiederanstieg besteht also eine vollständige Hemmung der Erythropoese

Die Erythrozytengrösse in der Regenerationsphase der APH Anämie

a) Messung des Retikulozytendurchmessers Bereits bei Durchmusterung des Brillantkresylblaupräparates erkennt man, dass die Retikulozyten auffällig gross sind, grösser als die Retikulozyten im steady state

Darunter befinden sich besonders in den ersten 10 Tagen nach APH zahlreiche



Bei 7 Kanin[illegible]

während des

[illegible]

durchmesser stimmt zeitlich mit der Zunahme der relativen (‰) Retikulozytenzahlen überein [8] Wegen der raschen Abnahme der Erythrozytenzahl in dieser Versuchsphase stellen aber nur die absoluten Retikulozytenzahlen (Retik./mm³) ein Mass für die Zellneubildung

dar. Ihr höchster Wert wird regelmässig später als der maximale Retikulozytendurchmesser erreicht.

Nach 12–14 Tagen ist die Anämie wieder ausgeglichen; in den folgenden Tagen treten Retikulozyten auf, deren Durchmesser kleiner ist als im steady state. Ganz allmählich nimmt die Retikulozytengrösse dann wieder zu, hat aber in manchen Fällen bei Versuchsende (28 Tage nach APH) den Ausgangswert noch nicht erreicht. Die Produktion kleiner Erythrozyten in der Spätphase der Regeneration ist *nicht Folge eines inzwischen eingetretenen Eisenmangels. Denn die* Tiere erhielten jeden 4. Tag 30 mg FeIII i.m.

b) Messung des mittleren Zellvolumens (MCV). Während wir mit den Retikulozyten immer die neu in das Blut eingeschwemmten Erythro-

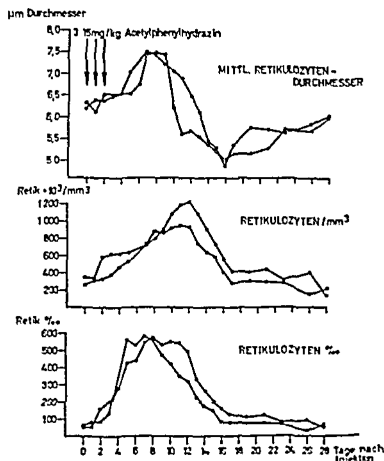


Abb. 2. Mittlerer Retikulozytendurchmesser und Retikulozytenzahl bei APH-Anämie.

Tabelle 11 Zahl und mittlerer Durchmesser der Retikulozyten nach Phenylhydrazinhämolyse

Tag	K 97			K 98			K 99			K 100			K 101		
	‰	pro mm³ × 10³	Durchm. µm	‰	pro mm³ × 10³	Durchm. µm	‰	pro mm³ × 10³	Durchm. µm	‰	pro mm³ × 10³	Durchm. µm	‰	pro mm³ × 10³	Durchm. µm
0	52	370	5,6	73	125	6,7	33	189	6,4	53	507	6,2	44	286	5,6
5	744	427	6,1	7,6	740	6,6	241		6,4	632	675	7,1	507	533	6,1
7	415	812	8,0	483	933	6,9	640	1 108	7,4	488	650	6,9	522	585	7,4
8	517	1 255	6,8	490	1 157	6,8	595	1 327	7,3	402	677	6,7	411	670	6,5
9	445	1 210	7,1	368	1 015	6,7	332	920	6,5	290	603	6,6	290	571	5,9
10	387	1 174	6,8	259	740	6,2	285	802	6,4	233	558	6,5	262	558	5,9
15	114	438	5,7	153	675	5,4	145	578	5,5	154	613	5,8	139	538	5,8
17	108	433	5,5	91	370	6,0	51	217	6,3	88	365	5,7	107	460	5,6
19	69	291	5,8	55	258	6,3	57	2 9	6,4	72	312	5,6	41	189	6,2

zyten oder zumindest solche messen, deren Entstehungszeit zeitlich eng umschrieben ist, repräsentiert der MCV-Wert immer alle bis zu diesem Zeitpunkt produzierten und noch im Blut kreisenden Zellen.

Wie schon von früheren Autoren [17, 25] beschrieben, tritt nach grossen APH-Dosen (45–60 mg/kg) eine schwere Anämie (1,0–1,2 Mill. Ery./mm³), nach geringeren Dosen (20–30 mg/kg) eine mittelschwere Anämie (2,8–3,0 Mill.Ery/mm³) auf. Vom Schweregrad der Anämie wiederum ist die Grosse der in der Regenerationsphase neugebildeten Erythrozyten abhängig. Dies geht aus Abbildung 3 hervor, in der bei 26 Versuchen der hochste gemessene MCV-Wert gegen die niedrigste Erythrozytenzahl aufgetragen ist.

Aus den fortlaufenden MCV-Bestimmungen ergibt sich (Abb. 4): von einem Ausgangswert von etwa 70 μm^3 steigt der MCV-Wert bei den schweren Anämien auf das Doppelte (136–141 μm^3), bei den mittelschweren Anämien auf das 1½fache (102–105 μm^3) an. Bei den schweren Anämien finden sich die maximalen MCV-Werte zwischen dem 6. und 9. Tag nach APH; aber bereits am 10.–12. Tag liegen die

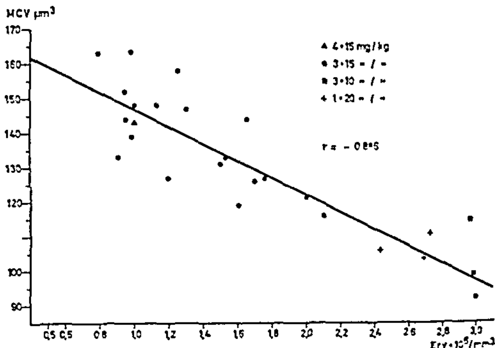


Abb. 3 Abhängigkeit des MCV in der Regeneration vom Grad der Anämie

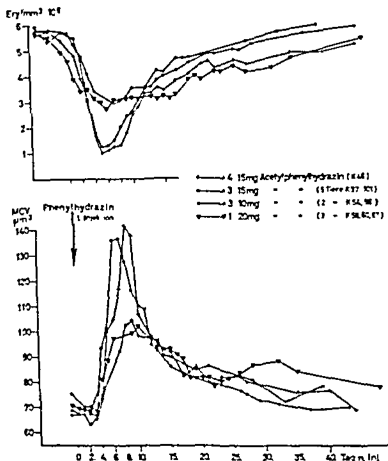


Abb. 4 Erythrozytenzahl und MCV in der Regenerationsphase der APH-Hämolyse

MCV-Werte wieder um 100 μm^3. Dies beweist noch nicht, dass die Makrozyten mit dem doppelten Zellvolumen innerhalb dieser kurzen Zeit zugrunde gegangen sind. Denn zu diesem Zeitpunkt sind die neugebildeten Erythrozyten normal gross oder bereits mikrozytär (Abb 2, Tab II). Der rasche Rückgang des MCV ist deshalb sicher zu einem erheblichen Teil durch die Mischung einer normal grossen mit einer makrozytären Population bedingt. Im weiteren Verlauf nehmen die

MCV-Werte allmählich ab, da die Ausschwemmung relativ kleiner Erythrozyten anhält. Erst nach etwa 40 Tagen ist der Ausgangswert des MCV wieder erreicht [25].

Die Erythrozyten- und MCV-Kurven von K 58, 60, 67 weichen von dem geschilderten Verlauf ab. Bei diesen Tieren hatten wir an die APH-Hämolyse vom 11.–18. Tag eine «Verdünnungsanämie» [9] durch Infusionen grosser Mengen eines Plasmaexpanders angeschlossen. Deshalb steigt die Erythrozytenzahl während dieser Zeit nicht an, das mittlere Zellvolumen nimmt nochmals gering zu

c) Messung der Volumenverteilungskurve (VK). Im Gegensatz zum MCV bietet die Messung der VK die Möglichkeit, Änderungen in der Grossenverteilung und das Auftreten und Verschwinden von Zellen eines umschriebenen Volumenbereiches zu erfassen. Da sich während des Versuchs die Erythrozyten-Gesamtzahl stark ändert, muss die Auswertung der VK über die bisher ausschliesslich übliche Beurteilung der prozentualen VK hinaus auch die absoluten Werte (Ery/mm³) innerhalb jeder Grössenklasse berücksichtigen.

Von «absolut» sollte streng genommen nur in bezug auf die Gesamtzahl aller zirkulierenden Erythrozyten gesprochen werden. Wegen der weitgehenden Konstanz des Blutvolumens bei akut verlaufenden Anämien [29, 34, 38, 48] kann aber ohne grössere Fehler die Erythrozytenkonzentration (Ery/mm³) als Mass für die Gesamtheit der Erythrozyten angesehen werden

Abbildung 5 gibt eine Verlaufsuntersuchung derartiger absoluter VK wieder, aus der Serie, bei der Actinomycin D 5 Tage nach der 1. APH-Injektion gegeben wurde. Sämtliche VK sind im gleichen Massstab aufgetragen, wodurch das Verschwinden und Wiederauftreten der Erythrozyten in den einzelnen Volumenklassen klar zur Darstellung kommt.

In den ersten Tagen nehmen die Zellen aller Grössen stark ab, am 7.11. ist der Normozytengipfel (bei 57 μm^3) verschwunden. Die erste makrozytäre Regeneration ist am 4.11., deutlicher am 5.11. zu erkennen (Doppelgipfel). In der Phase der Actinomycin-D-Hemmung (7.–11.11.) nimmt die Kurvenhöhe ab, der breite Kurvengipfel im Makrozytenbereich (102 μm^3) wandert allmählich nach links (11.11. 89 μm^3). Mit Wegfall der Actinomycinwirkung (ab 15.11.) nimmt die Höhe der Kurven wieder zu, der Gipfel verschiebt sich weiter nach links, ab 28.11. liegt der Kurvengipfel erstmals links vom Ausgangswert, erst am 2.1. findet sich wieder die normale VK.

Eine selektive Messung der VK der jeweils neugebildeten Erythrozyten, analog zur Durchmesserbestimmung der Retikulozyten, ist

mit dem verwendeten Anreicherungsverfahren nicht möglich. Im oberen Drittel des Erythrozytensedimentes sind aber die Retikulozyten und jüngsten Erythrozyten stark angereichert; ihre prozentuale VK ist in Abbildung 6 dargestellt.

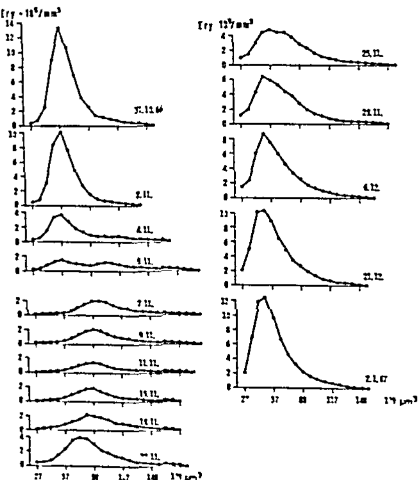


Abb. 5. Absolute Volumenverteilungskurven bei Phenylhydrazinhämolyse. APH 31.10., 1.11., 2.11. je 15 mg/kg. Actinomycin D 5.11., 9.11. je 100 µg/kg

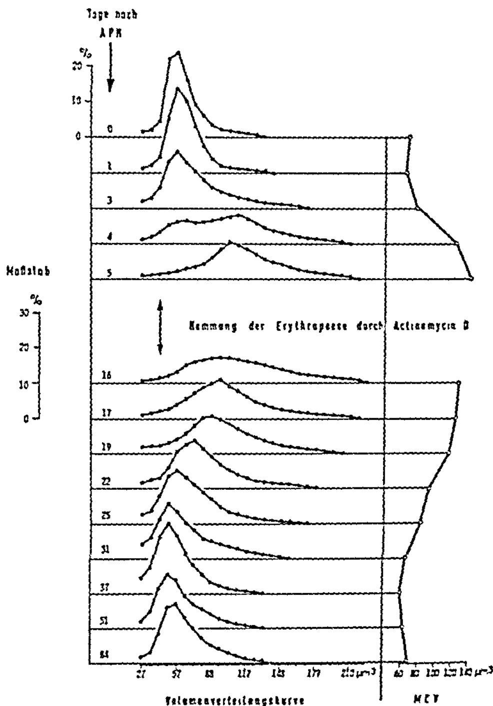


Abb. 6 Prozentuale Volumenverteilungskurve und mittleres Zellvolumen (MCV) des oberen Drittels des Erythrozytensedimentes.

3. Tag: Zunahme der Makrozyten. 4. Tag: Doppelgipfel. 5. Tag: Normozyten stark vermindert, Makrozytengipfel (102 μm^3). Mit Nachlassen der Actinomycinwirkung setzt die Regeneration erneut makrozytär ein. Im weiteren Verlauf verschwinden die Makrozyten aus der oberen Schicht, der Kurvengipfel wandert nach links, über die Gipfellage der Ausgangskurve hinaus. Die Parallele zu den Retikulozytenmessungen (Abb. 2) ist evident.

Die Regeneration nach APH-Hämolyse ist somit makrozytär, solange eine schwere Anämie besteht. Über ein normozytäres Zwischenstadium wird sie dann fast mikrozytär. Dieser Umschlag wird besonders deutlich bei Gegenüberstellung der VK von Vollblut, oberem und unterem Drittel des Erythrozytensedimentes aus der Früh- und der Spätphase des Versuchsablaufs (Abb. 7).

Am 5. T[illegible]
aus der 7. [illegible]
(Abb. 7b) [illegible]
Regenerat [illegible]
fast identisch mit dem arithmetischen Mittel aus oberem und unterem Drittel des Erythrozytensedimentes.

Um die Grössenänderungen der Erythrozyten besser erkennen und Zellstörungen von Zellschrumpfung nach Möglichkeit voneinander abgrenzen zu können, haben wir die absoluten VK zu verschiedenen Zeitpunkten aufgezeichnet und für jeden Messpunkt der VK die Differenz der Erythrozyten berechnet. In den Abbildungen 8 und 9 sind die so ermittelten *Differenzkurven* für einen Versuchsablauf dargestellt. Die Ergebnisse der übrigen Versuche sind in Tabelle IV zusammengefasst. In den Abbildungen sind links die absoluten VK (Ery/mm³), rechts die Differenzkurven dargestellt. Die zeitlich erste VK ist jeweils mit Punkten (•), die zweite mit offenen Kreisen (○) gekennzeichnet.

Abbildung 8 zeigt die Grössenänderungen vom Beginn der APH-Vergiftung bis zum Eintritt der vollständigen Erythropoesehemmung. Abbildung 8a umfasst das Stadium der reinen Hämolyse, die negative Differenzkurve stellt das genaue Spiegelbild einer normalen VK dar. [illegible]

[illegible] der makrozytären Regeneration, sie überdeckt die auch in dem Volumenbereich von mehr als 83 μm^3 fortbestehende Hämolyse. Die Änderung der Gesamt-Erythrozytenzahl ist die Resultante von Produktion und Destruktion; die Abnahme der Gesamtzahl beträgt

690×10^3, die Differenzkurve zeigt dagegen eine Zellzerstörung von 1423×10^3 allein im Volumenbereich bis 88 μm^3 an. Die Zellzerstörung ist also mehr als doppelt so gross wie die Abnahme der Erythrozytenzahl.

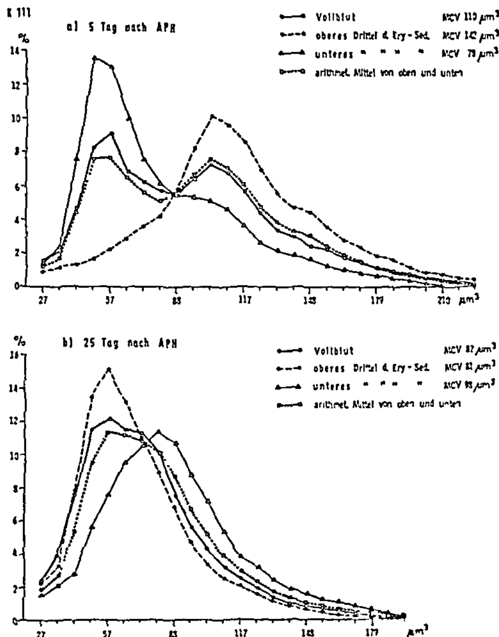


Abb. 7. Prozentuale Volumenverteilungskurven von Vollblut, oberem und unterem Drittel des Erythrozytensedimentes, *a*) in der Frühphase, *b*) in der Spätphase der APH-Anämie.

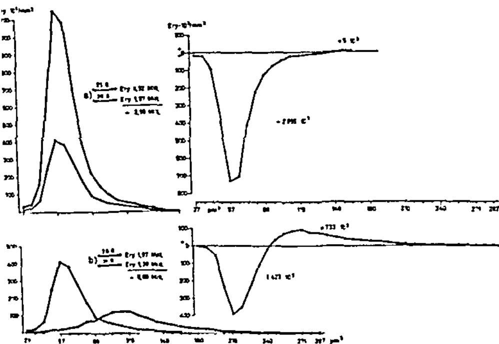


Abb. 8 Volumenverteilungskurven und Differenzkurven in der ersten Phase der APH Hämolyse Am 25., 26. und 27. 8. wurden je 15 mg/kg APH injiziert.

Die Änderungen der VK während der anschliessenden achttägigen Erythropoesehemmung sind in Abbildung 9 dargestellt, unterteilt in 3 kurze Zeitintervalle, während derer sich die VK immer mehr nach links verschiebt. Die Abnahme der Erythrozytenzahl beträgt während des gesamten Zeitraums 320×10^3, aus den negativen Anteilen der 3 Differenzkurven ergibt sich dagegen eine Abnahme von $305+200+130 = 635 \times 10^3$. Im positiven Bereich der Differenzkurven ist eine Zellzunahme von $191+84+38=313 \times 10^3$ nachweisbar, obwohl während dieser Zeit keine Erythrozyten aus dem Knochenmark in die Blutbahn eintreten. Das heisst, dass eine entsprechende Zahl von Makrozyten zu kleineren Zellen geschrumpft sein muss. Der positive Bereich der Differenzkurven gibt somit den Anteil wieder, den die Zellschrumpfung am Rückgang der Makrozyten hat. Der Anteil der Hämolyse ergibt sich aus der Differenz zwischen dem negativen und

dem positiven Bereich der Differenzkurven. Zunächst überwiegt die Schrumpfung (Abb. 9a), später die Hämolyse (Abb. 9c).

Die Abgrenzung dieser Intervalle wurde nach Berechnung der absoluten VK so vorgenommen, dass charakteristische Häufigkeitsänderungen in der Grössenverteilung zusammengefasst wurden Sie sind deshalb nicht gleich lang, zum besseren Vergleich wurden deshalb in Tabelle III Schrumpfung und Hämolyse für jedes Intervall auf den Durchschnittswert pro 24 Std umgerechnet Die Hämolyserate zeigt in den 3 Intervallen keine Tendenz in Richtung einer Zu- oder Abnahme, die Schrumpfungsrate ist im 1 Intervall(a) am grössten und sinkt dann kontinuierlich ab Dementsprechend nimmt der MCHC-Wert im 1 Intervall zu Die MCV-Abnahme in Intervall a) ist mehr durch Schrumpfung als durch Hämolyse [illegible] wiegend werden die besonders grossen Erythrozyten hämolysiert (MCV-Abnahme)

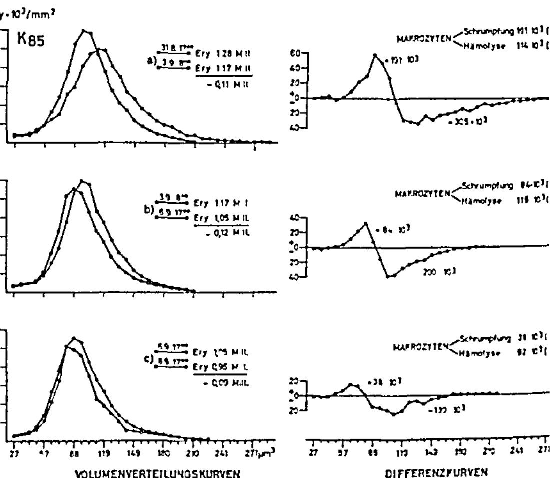


Abb 9 Absolute Volumenverteilungskurven und Differenzkurven während einer 8tägigen Hemmung der Erythropoese durch Actinomycin D

Wie aus Abbildung 9 hervorgeht, erfasst die Hämolyse in den Intervallen b) und c) auch Zellen einer Grössenklasse, deren Häufigkeit zuvor (infolge Schrumpfung grösserer Zellen) zugenommen hatte *Ein Teil der Makrozyten schrumpft also zunächst, um dann doch noch hämolysiert zu werden*

Tabelle IV zeigt Schrumpfung und Hämolyse während der ersten 48 Std nach vollständiger Erythropoesehemmung bei den übrigen Versuchstieren In allen Versuchen ist die Abnahme der Makrozyten nicht nur durch Hämolyse, sondern auch durch eine Zellschrumpfung verursacht

Tabelle III

Intervall	Std.	Schrumpfung pro 24 Std Ery × 10⁴	Hämolyse pro 24 Std. Ery × 10⁴	MCV µm³	MCHC %	Hbg PS
a) 3,8				150	25,0	36,9
3,9	63	73	43	124	30,5	36,9
b) 3,9				124	30,5	36,9
6,9	81	25	34	115	30,5	34,9
c) 6,9				115	30,5	34,9
8,9	48	19	46	97	32,2	31,2

Schrumpfungen und Hämolyse pro 24 Std in 3 Intervallen (a, b, c)

Tabelle IV Schrumpfung und Hämolyse in den ersten 48 Stunden der vollständigen Erythropoesehemmung

Kan Nr	Makrozyten Abnahme × 10⁴	Schrumpfung Ery × 10⁴	Schrumpfung % der Makrozyten-Abnahme	Hämolyse Ery × 10⁴	Hämolyse % der Makrozyten-Abnahme	Hbg PS	MCV µm³	MCHC %
K 54	137	47	32	90	68	32,0→31,0	134→124	24,3→25,0
K 103	310	54	17,4	256	82,6	32,0→32,0	119→109	26,9→30,0
K 111	170	140	82,5	30	17,7	34,0→33,5	125→117	28,2→29,7
K 118	118	61	51,6	57	48,4	40,0→37,0	147→134	27,5→29,1

Diskussion

Die Regeneration der Erythropoese nach APH-Hämolyse ist gekennzeichnet durch das Auftreten makrozytärer Retikulozyten mit dem 1½- bis 2fachen Volumen normaler Erythrozyten. Je schwerer die Anämie, umso ausgeprägter ist die Makrozytose. In zahlreichen Arbeiten haben sich besonders STOHLMAN *et al.* [18, 84, 89, 92] sowie BORSOOK *et al.* [13, 14, 15] um die Klärung dieses Phänomens bemüht. Es darf danach als gesichert gelten, dass die Makrozytose dadurch zustande kommt, dass bei starker Stimulierung eine (oder zwei) Reifeteilungen übersprungen werden («skipped division»), die Retikulozyten also durch Entkernung der polychromatischen (oder basophilen) Normoblasten entstehen. Die vorzeitig ausgeschwemmten Zellen haben eine verkürzte Lebenszeit [5, 18, 69, 84, 103], die Makrozytose ist nach wenigen Tagen nicht mehr nachweisbar. Dieser «Panikmechanismus» der Erythropoese [84] vermag immerhin, den bedrohlichen Grad der Anämie zu überwinden. Er ist grundsätzlich von der normalen Zellbildung mit Kernausstossung der ausgereiften orthochromatischen Normoblasten und der Ausschwemmung von Erythrozyten mit normaler Lebenszeit zu unterscheiden. Wir haben deshalb diesen Modus der Zellbildung, der in seiner Auswirkung auf die periphere Erythrozytenzahl zwischen normaler (effektiver) und der ineffektiven Erythropoese [30, 41] steht, als semi-effektive Erythropoese bezeichnet [7, 10]. Er kommt auch, allerdings in quantitativ unbedeutendem Ausmass, bei ausgeglichener Zellbildung vor [57, 63, 86].

Die vorliegenden Untersuchungen haben gezeigt, dass das rasche Verschwinden der Makrozyten nicht nur auf einer Hämolyse, sondern auch auf einer Zellschrumpfung beruht. Ein indirekter Beweis hierfür ergibt sich aus dem Vergleich der mittleren Retikulozytendurchmesser mit der absoluten Retikulozytenzahl während des gesamten Versuchsablaufs. Der Retikulozytenanstieg am 5.–8. Tag nach APH kommt nicht allein durch die zunehmende Zellausschwemmung zustande, sondern auch dadurch, dass die Reifungszeit im Blut verlängert ist [31, 77], was an einer erheblichen Linksverschiebung der Reifestadien der Retikulozyten zu erkennen ist. Dadurch kommt es zu einer Kumulation, die maximalen Retikulozytenzahlen werden regelmässig nach dem Maximum der Retikulozytendurchmesser gefunden. In Anbetracht der Kumulation ist dies so zu erklären, dass die ausreifenden Retikulozyten an Grösse abnehmen [72].

Der direkte Beweis für die Schrumpfung makrozytärer Erythrozyten wurde durch Messung der Volumenverteilungskurven (VK) und Berechnung der Differenzkurven erbracht. Die Interpretation dieser Ergebnisse erfordert eine kritische Bewertung der Messmethode.

Seit der grundlegenden Arbeit von BRECHER *et al.* [14] wurde bis in jüngste Zeit [24, 96] angenommen, dass die rechtsschiefe VK, die wir bei Messung mit dem Coulter Counter oder ähnlichen Geräten erhalten, der realen Grössenverteilung der Erythrozyten entspricht. Zahlreiche Autoren haben sich mit dieser Rechtsschiefe auseinandergesetzt [24, 36, 46, 65, 66, 70, 96, 98, 104], die im Gegensatz zur Normalverteilung der Erythrozytendurchmesser steht. BULL [19] fand bei methodischen Modifikationen der üblichen Grössenmessung eine Gaussche Normalverteilung. THOM [94, 95] konnte in ausgedehnten Versuchen nachweisen, dass die Stromdichte am Rand der Kapillaröffnung grösser ist als in der Achse der Messöffnung, so dass Erythrozyten, die über den Rand der Öffnung in die Messkapillare einströmen, zu gross registriert werden. Bei Verwendung einer modifizierten Kapillaröffnung, die es erlaubt, die Zellen ausschliesslich im Zentralstrahl zu messen, fand auch er eine Gaussche Normalverteilung der Erythrozytenvolumina.

Bei den in den Abbildungen 5–9 dargestellten VK ist, ebenso wie bei allen früheren Messungen der VK bei APH-Hämolyse [17, 18, 90], dieser methodisch bedingte Messfehler [illegible]



In allen Versuchen ist nach vollständiger Erythropoesehemmung eine erhebliche Zunahme normozytärer Erythrozyten nachweisbar, die nur durch Schrumpfung von Makrozyten entstanden sein können. Der Anteil der Schrumpfung am Rückgang der Makrozyten ist in den ersten Tagen am grössten, ein Teil der Makrozyten, die zunächst geschrumpft waren, wird in den folgenden Tagen dann doch noch hämolysiert.

Ausser Schrumpfung und Hämolyse ist als 3. Möglichkeit des Schicksals der Makrozyten die von WEICKER [99] diskutierte Retikulozytenteilung in Betracht zu ziehen. Sie ist aufgrund der Differenzkurve in Abbildung 9a und der Befunde bei den übrigen Versuchstieren auszuschliessen. Zu Beginn der Knochenmarkshemmung wurden 6,5‰ Retikulozyten gezählt, am Ende des 1. Intervalls (3.9.) 0. Wäre die Abnahme der Makrozyten durch eine Teilung verursacht, dann wäre eine Verdoppelung der Erythrozyten mit halbem Volumen und dadurch ein erheblicher Anstieg der Gesamtzellzahl zu erwarten. Die Hypothese der Retikulozytenteilung ist bereits von LOWENSTEIN [64] widerlegt worden.

Die Ergebnisse zeigen, dass den Zellgrössenänderungen bei der Regeneration wesentlich kompliziertere Vorgänge zugrunde liegen, als ursprünglich [17, 88] angenommen worden war. Geradezu dramatische Vorgänge von Zellproduktion, Hämolyse und Schrumpfung

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Summary

After experimental haemolytic anaemia caused by acetylphenylhydrazine there is a restoration of erythropoiesis showing a markedly macrocytic character. The more severe the anaemia the larger are the erythrocytes formed. The macrocytic cell population is replaced within a few days by a normocytic one. When the number of erythrocytes has regained the initial value cells are produced for a time which are smaller than normal. After a few days nothing more remains to be seen of the macrocytosis of the first regeneration phase. By inhibiting erythropoiesis with actinomycin D at the peak of reticulocytosis it was shown by means of the volume distribution curve that some of the macrocytes undergo haemolysis whereas others shrink to the size of normal erythrocytes. The continuous rise in the number of erythrocytes in the regenerative phase after anaemia thus conceals complex processes of overproduction, haemolysis and cell shrinkage. These results are discussed in the light of STOHLMAN's model of erythropoiesis.

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Diese Hypothese, die die Hämoglobinkonzentration in den Ery[illegible] zyten in den Mittelpunkt der Erythrokinetik stellt, ist u. E. von [illegible] sonderem Interesse in einem grosseren biologischen Zusammenh[illegible] vergleicht man das mittlere Zellvolumen (MCV), den Hämoglobin[illegible] gehalt (Hb_E) und die Hämoglobinkonzentration (MCHC) bei [illegible] grosseren Zahl von Saugetier-Erythrozyten, dann findet man [illegible] verschiedene MCV- und Hb_E-Werte, während die MCHC-Werte [illegible] gering schwanken. PONDER [74] hat diese Indices von 21 verschieden[illegible] Saugetieren zusammengestellt: die Werte des MCV lagen zwischen 14 und 84 μm^3, die des Hb_E zwischen 7 und 26 pg, die MCHC-W[illegible] schwankten dagegen nur zwischen 30 und 35%. JUNG [47] hat [illegible] Hinweis auf die Studien von PERUTZ [73] darauf hingewiesen, dass [illegible] Hämoglobinmolekul zur optimalen Funktion des Gasaustausches [illegible] Rotationsraum braucht; die Grenzkonzentration, bei der [illegible] Hämoglobinmolekul gerade noch dieser Rotationsraum zur [illegible] fugung steht, beträgt 34%. Bei dieser Konzentration besitzt [illegible] Erythrozyt die hochste Sauerstoffkapazität. Eine Steigerung [illegible] Hämoglobinkonzentration wurde die hohe Reaktionsgeschwindigkeit des Farbstoffs im Zellinnern, aber auch das Diffusionsvermogen [illegible] Gase einschränken. Der gleiche Parameter, der sowohl in der Säu[illegible] tierreihe als auch beim Menschen unter physiologischen und pa[illegible] gischen Bedingungen weitgehend konstant ist [3, 12, 23, 39, 71] [illegible] damit im Mittelpunkt der Vorstellungen uber die Regulation [illegible] Erythropoese, nach der die Erythropoetin-gesteuerte Hämo[illegible] synthese bei Erreichen einer kritischen Konzentration die [illegible] Synthese hemmt, eine weitere Zellteilung verhindert und die [illegible] schwemmung der Retikulozyten einleitet.

Zusammenfassung

Nach experimenteller hämolytischer Anämie durch Acetylphenylhydrazin [illegible] zu einer ausgeprägt makrozytären Regeneration der Erythropoese Die [illegible] Erythrozyten sind um so grösser, je schwerer die Anämie ist Die makrozytäre [illegible] wird nach wenigen Tagen von einer normozytären abgelost. Wenn die Ery[illegible] den Ausgangswert wieder erreicht hat, werden vorübergehend Zellen gebildet, die [illegible] als normal sind Die Makrozytose der ersten Regenerationsphase ist bereits [illegible] Tagen nicht mehr nachweisbar Bei Hemmung der Erythropoese durch [illegible] auf dem Hohepunkt der Retikulozytose konnte mit Hilfe der Volumen[illegible] nachgewiesen werden, dass die Makrozyten teilweise hämolysiert werden, teilweise [illegible] auch zu normal grossen Erythrozyten schrumpfen. Hinter dem stetigen Anstieg der Ery[illegible] zytenzahl in der Regenerationsphase der Anämie verbergen sich somit komplexe [illegible] von Mehrproduktion, Hämolyse und Zellschrumpfung Die Ergebnisse werden im Zu[illegible] menhang mit dem Modell der Erythropoese von STOHLMAN diskutiert.

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Incorporation of ³H-Uridine and ³H-Leucine in Erythroid Cells of Normal and Anemic Rabbits[1]

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It is well known that in the anoxic tissue of anemic animals a humoral factor or erythropoietin is generated, which stimulates the stem cells [4] It has been observed in anemic rabbits [5] and in hypertransfused mice [7] that this factor operates by accelerating the differentiation of stem cells to proerythroblasts

Already in 1959 MATOTH *et al* [11] reported some results on the effect of erythropoietin on mitosis They incubated in normal serum bone marrow cells of rabbits made anemic by bleeding and observed that the mitotic rate of anemic bone marrow cells was higher than that of normal ones in the same conditions The authors concluded that anemic bone marrow becomes hyperactive because of the erythropoietin stimulation *in vivo* In order to see whether this increased mitotic activity is connected with changes in the metabolic pathway, we have undertaken a study of ³H-uridine and ³H-leucine incorporation in erythroid cells of normal and anemic rabbits

Methods

Two normal and two anemic rabbits weighing approximately 3 kg have been used The animals were made anemic by phenylhydrazine, following the method of BORSOOK *et al* [2] In the anemic animals the reticulocyte count was 70–80% and the bone marrow smears stained with May Grünwald-Giemsa showed erythroid hyperplasia

Bone marrow was obtained from the femur In each experiment 1 ml of bone marrow was suspended in 1 ml of Eagle's medium and then incubated at 37° C in silicon covered conical tubes.

[1] The experimental part of this work has been carried out at the Institute of Animal Morphology, Free University of Brussels

Four types of experiments have been performed for each animal. (1) The rate of nuclear

^{3}H-uridine, since it acts on the DNA-dependent RNA [9] (3) The rate of protein synthesis was studied by adding ^{3}H leucine, at 5 μc/ml concentration (spec. act. 500 mc/mM), to the culture medium [3] (4) Since puromycin releases the labelled protein from ribosomes [1, 12] we studied the ribosomal activity by adding puromycin at 1 μg/ml concentration to a replica tube with ^{3}H leucine in the medium. Following 3 hours of incubation some smears were made on gelatin coated slides and fixed in methanol. The slides were coated with nuclear emulsion Ilford K 5, and, after the required exposure time at 4° C, autoradiographs were developed and stained with May-Grünwald-Giemsa, for differential counts.

For each stage of erythroid line, a minimum of 100 elements were examined. The number of grains was counted visually and the labelling index was calculated in the proerythroblasts, basophil, polychromatic and orthochromatic erythroblasts in the slide of each experiments.

Results

The results are shown in figures 1–3. As can be seen, the incorporation of ^{3}H-uridine and ^{3}H-leucine in erythroid cells decreases as maturation proceeds [8–6], the decrease being however smaller for ^{3}H-leucine incorporation. From the results of figure 3, one can see that the uptake of ^{3}H-uridine and ^{3}H-leucine is considerably higher in

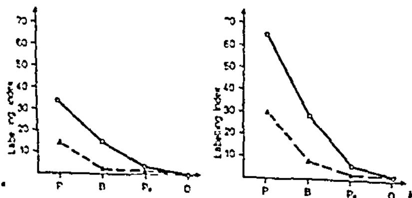


Fig. 1a. Normal rabbits, *b* anemic rabbits. ——— ^{3}H-uridine uptake, – – – ^{3}H-uridine uptake in presence of actinomycin, *P* proerythroblasts, *B* basophil erythroblasts, *Pe* polychromatic erythroblast, *O* orthochromatic erythroblasts.

* Actinomycin D was a gift from Merck-Sharp-Dohme.

proerythroblasts of anemic than in those of normal animals In more mature erythroblasts the above effect is less conspicuous

Actinomycin D reduces the incorporation of the ^{3}H-uridine precursor by about 50% (fig 1), especially in immature stages Puromycin acts on ^{3}H-leucine incorporation of all stages of erythroid line, but its effect is mainly exerted on the proerythroblasts of anemic animals (fig 2)

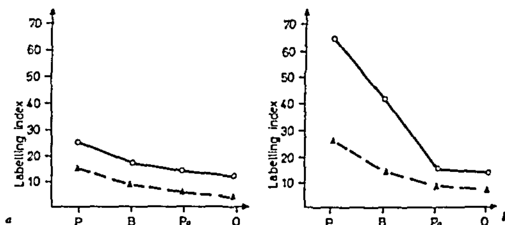


Fig 2a Normal rabbits, *b* anemic rabbits ——— ^{3}H leucine uptake, - - - ^{3}H leucine uptake in presence of puromycin Abbreviations see figure 1

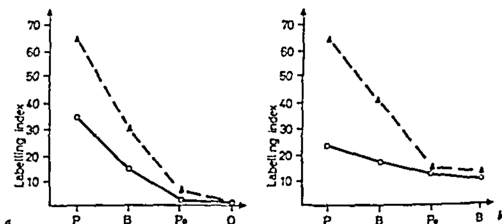


Fig 3a ^{3}H uridine uptake, *b* ^{3}H leucine uptake ——— Normal rabbits - - - anemic rabbits. Abbreviations see figure 1

Discussion

The results show that the rate of nucleic acid and protein synthesis of erythroid cells is higher in anemic animals Actinomycin D reduces the increase of nucleic acid synthesis It is known that actinomycin D acts on the DNA dependent RNA [9] TORELLI *et al.* [15] reported that the rapidly degradeted RNA observed in immature erythroid cells is an unstable messenger RNA synthesized in the more immature stages, that does not act as 'a template' for protein synthesis [16]

Early experimental observations [13] indicate that erythropoietin containing cultures of bone marrow cells show an increase in the rate of ^{14}C-uridine uptake during 4 hours of incubation, while in the control cultures a decrease of this rate is observed Actinomycin D added to the culture prevented the above mentioned increase It thus appears that erythropoietin acts on the actinomycin sensitive RNA fraction, i e m RNA [9] One may thus assume that the same m RNA fraction is increased in the erythroid cells of anemic animals, for the erythropoietin stimulation *in vivo*

Since protein synthesis is mediated through m RNA [10] and it provides differentiation, it seems reasonable to conclude that in anemic animals the effect of differentiation may be similar to the effect on mitosis reported by MATHOT *et al* [11] According to the experimental results mentioned in the introduction [5] one can believe that erythropoietin is the factor mainly involved in stimulative action on the erythroid cells of anemic animals

The mechanism of action of erythropoietin has been recently studied by STOHLMAN *et al* [14], who concluded that it acts not only by differentiating the precursor cells, but also by accelerating the hemoglobin synthesis in the differentiated erythroid precursor still capable of synthesizing RNA In agreement with this model, the increased metabolism found in the present experiments can be related to the stimulative action of erythropoietin on the proerythroblasts of anemic animals

Acknowledgements. The author expresses her gratitude to Drs. A. FICQ, and F GAVOSTO for their help and criticism.

Summary

The incorporation of ^{3}H-uridine and ^{3}H-leucine in erythroid cells of normal and anemic rabbits has been studied by autoradiography. The rate of m RNA and protein synthesis is higher in proerythroblasts of anemic animals. The increased metabolism can be related to the stimulative action of erythropoietin on the cells of anemic animals.

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Hereditary Methemoglobinemia due to Diaphorase Deficiency

Report of a Case of Heterozygote Presenting with Cyanosis After Birth

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Methemoglobinemia is characterized by a diffuse slate gray cyanosis and caused by excessive amounts of methemoglobin in the erythrocytes. This disorder may result from a) exposure to oxidizing agents and drugs such as nitrates, aniline derivatives, resorcin, and sulfonamides b) genetically determined deficiency of one of the enzymes required for reduction of methemoglobin to hemoglobin, and c) genetically determined abnormalities in the structure of hemoglobin molecule (HbM) which increase the susceptibility to oxidation and decrease the susceptibility to reduction

About 250 cases of hereditary methemoglobinemia, presumed or proved to be due to DPNH methemoglobin reductase (diaphorase) [illegible] been reported since 1844 [11] The condition is trans [illegible]

zygotes are not cyanotic and have norm [illegible] of methemoglobin [2] with moderate degrees of enzyme deficiency Heterozygotes with high concentrations of methemoglobin are uncommon however during the last years a small number of cases have been described [3 9 15 17] We have recently had an opportunity in observing a heterozygote presenting with cyanotic attacks and a high level of methemoglobin after birth who at the same time had a hypocalcemia

Methods

Routine hematological and biochemical investigations were performed by standard [illegible]

Methemoglobin was determined by the standard method of Evelyn and Malloy [6] For the determination of DPNH methemoglobin reductase the method of Scott [18] was used The conventional methods for the evaluation of the reduction of methemoglobin in intact erythrocytes [14] and for the determinations of glycolytic enzymes in hemolysates [4] have been described elsewhere The capacity of methemoglobin reduction during red cell incubation with lactate is expressed in mg reduced hemoglobin per 100 mg methemoglobin per hour For the determination of reduced glutathione the method of Stevenson [19] was used The presence of HbM was excluded by starch block electrophoresis pH 6 5 pH 7 0 and pH 8 6 and by spectral analyses of methemoglobin and methemoglobin-cyanide

Case History

B D (J No 8595/66), a male infant of Italian origin, born in 1966 was admitted to us at 12 days old for further investigations of his cyanotic attacks He is only child and was born at full term, weighing 4 5 kg, after a normal pregnancy There was no history of drug-taking exposure to toxic agents which may enhance the oxidation of hemoglobin, diarrhoea or hemolysis during pregnancy The infant appeared to be well after birth until 4 days old when he was noticed to have cyanosis of the lips which gradually became more prominent Two days later he developed dyspnoea However, the cyanosis and breathlessness seemed to be transiently improved by oxygen On admission he had cyanosis of the lips, hands and feet which was more marked when the child was crying Apart from the irritability and jitteriness there was no positive evidence, clinically nor radiologically, of heart or lung diseases Preliminary investigations showed Hb 19 g%, of which 12 5% methemoglobin, hematocrit 60%, serum Ca 6 8 mg%, blood sugar 97 mg% Further investigations on several occasions showed the following results

Methemoglobinemia The results are summarized in table I and figure 2

Hypocalcemia Serum Ca 6 8 mg% Phosphate 6 9 mg% Alk phosphatase 137 IU Serum protein 5 7 g% Blood urea 17 5 mg% Urinary Ca excretion 3 mg/kg body weight/24 h Urin phosphate excretion 34 7 mg/kg body weight/24 h Creatinin clearance 62 ml min

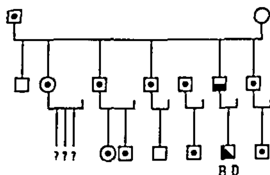


Fig 1 Genealogical tree of family

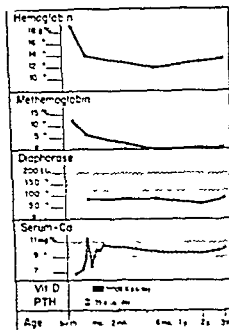


Fig. 2. Levels of hemoglobin, methemoglobin, diaphorase, and serum Ca in patient R. D.

Phosphate clearance 8.3 ml/min. Results of parathormone-test indicated the presence of hypoparathyroidism. X-rays of the skull normal. EEG negative.

Course (fig. 2). Without specific treatment the methemoglobin levels gradually fell from 12.5% (Hb 14 g%) at 12 days old to 6.5% (Hb 14 g%) at 4 weeks old, and to nil (Hb 12 g%) at 6 months old during which the cyanosis also disappeared. On admission the patient was given calcium i.v. on three occasions and the serum Ca rose to a maximum of 7.5 mg%. At 1 month old he was given vitamin D 20,000 units daily for a fortnight and following this the serum Ca became normalized.

Family history. The patient's mother stated that her husband has cyanosis of the lips, cheeks, and both hands when he is annoyed and when the weather is cold. Other members of the family on the paternal side are said to have similar phenomena. Unfortunately we are not in the position to investigate the rest of the family as the parents are not willing to disclose our findings to them. If these vague histories can be accepted as evidence of methemoglobinemia we may construct a family tree as shown in figure 1.

Discussion

Normally small amounts of methemoglobin are being constantly produced [16]. In normal adults methemoglobin levels average 0.82% of the total hemoglobin, with a range of 0 to 1.9% [6]. Slightly higher

Table 1 Results of investigations from the patient and his parents on different occasions.

	Patient				Father				Mother		
	July 1966	Dec 1966	August 1968	Sept 1969	July 1966	Dec 1966	August 1968	Sept 1969	July 1966	Sept. 1969	Normal values
Hemoglobin, g%	14	12	13	13.5	15	–	15.5	15.2	13	12.5	
Reticulocytes, %	2	2	3	2.4	–	–	2.3	2.9	–	4.5	
Hematocrit, %	40	–	40	41.0	–	–	49.0	48.0	–	39.0	
Methemoglobin %	6.5	0	0.69	0.81	0	0	0	0.61	0	0.47	
Diaphorase, IU	86	88	68	90.0	102	85	85.0	97.0	150	141	120–200
Hemoglobin M, %	0	0	0	0	0	0	0	0	0	0	
Capacity of methemoglobin reduction mg%/hour during incubation with lactate to hemoglobin	–	–	4.6	–	–	–	4.7	–	–	–	8–12
Glucose-6-phosphate dehydrogenase, IU	16	21	20	18.0	–	17	20.0	15.0	–	14.0	12–22
6 phosphogluconic dehydrogenase, IU	–	16	19	16.0	–	22	–	17.0	–	18.0	10–16
Glutathione reductase, IU	–	16	12	11.0	–	12	–	11.0	–	11.0	7–14
Hexokinase, IU	–		1.6	2.1	–	–	–	1.5	–	1.9	0.8–2
Phosphoglyceromutase, IU	–	–	34	68.0	–	–	–	54.0	–	73.0	20–38
Pyruvate kinase IU	–	–	40	34.0	–	–	–	39.0	–	42.0	24–56
Reduced glutathione, mg%/100 ml Ery		59	66	63.0	–	59	–	69.0	–	67.0	50–90

levels up to 4.4% of methemoglobin in newborn infants, particularly in prematures, have been found by KRAVITZ et al. [12]. In hereditary methemoglobinemia, homozygotes have levels of methemoglobin generally exceeding 10% and they may even be as high as 60 to 70% of the total hemoglobin in severely affected children. Our patient, a heterozygote, started to have cyanotic attacks at 4 days old. At 12 days old 12.5% of the total hemoglobin was methemoglobin. Heterozygotes presenting with cyanosis and high levels of methemoglobin are extremely rare and provide further evidence, that the deficiency in DPNH methemoglobin reductase is a heterogenous disorder. Our observation may be the third heterozygote known in the literature to show a high level of methemoglobin with cyanosis and reduced DPNH-methemoglobin reductase in the newborn period, the other two cases were published by RICHARD and MOINET [17] and HARPER et al. [9] respectively.

Antimalarial drugs have recently been observed in adults to produce methemoglobinemia in heterozygotes who otherwise have normal levels of methemoglobin but abnormal DPNH methemoglobin reductase [3]. In our case, the patient was said to have cyanosis at age 4 days. There was no relevant history during pregnancy and careful examination of the infant's environment (hospital nursery) for possible toxic agents and drugs which may give rise to methemoglobinemia was negative. Our investigations showed that in addition to methemoglobinemia the infant had hypocalcemia. At one month old his serum Ca was subnormal and 6.5% of the total hemoglobin was methemoglobin. After a fortnight course of vitamin D the serum Ca was normalized. The concentration of methemoglobin was then not determined but at 6 months old there was no methemoglobin detected and the serum Ca remained normal. Without specific treatment for his methemoglobinemia the levels of methemoglobin became normal. Could this be a coincidence or could hypocalcemia which causes no clinically detectable methemoglobinemia stress heterozygotes to produce significant methemoglobinemia in the newborn period? The answer to these questions requires further observations since this is the only case so far described.

Hereditary methemoglobinemia associated with various degrees of mental retardation has been reported in several cases [5, 7, 8, 10, 13, 20], and there has been controversy as to the cause of this association. In the case most similar to ours described by HARPER et al. [9] with

transient methemoglobinemia in a heterozygote for diaphorase deficiency a marked mental retardation with disturbed speech development and left hand preferance was observed. As possible causes the authors discuss an analogous enzyme deficiency in blood and brain; nervous system damage by hypoxia or by hyperbilirubinemia; or activation of other genes. However, none has mentioned hypocalcemia as a possible contributing factor to the neurological disability. Should we fail to recognize and alleviate the hypocalcemia in our patient, the child might have developed epileptic attacks which might or might not ultimately affect his mental state. The role of hypocalcemia in the association of methemoglobinemia and mental retardation is uncertain but certainly needs to be examined, as the latter arising from hypocalcemia is preventable.

Summary

hemoglobinemia in heterozygotes and as a contributing factor in the association of methemoglobinemia and neurological disorders is discussed

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Swiss Type Hereditary Persistence of Foetal Haemoglobin in a Case of Acquired Haemolytic Anaemia

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High concentrations of foetal haemoglobin (Hb F) in red blood cells has been found in several types of anaemias in adults. This may result from the diminution of β-chain synthesis (in β-thalassaemia) or from accelerated breakdown of labile β-chain variants of haemoglobin. The mechanisms responsible for the increase of Hb F concentration in other types of anaemias remain, however, uncertain.

On the other hand various types of hereditary persistence of Hb F synthesis were reported in which no evidence of accelerated haemolysis or anaemia has been found. Hb F contributes 14, 21 and 25% of total haemoglobin in heterozygotes for the Greek, Mongolian and African types respectively [1, 2]. In a case of homozygote of an African *type hereditary persistence* of Hb F, the latter constituted 100% of haemoglobin, yet anaemia did not occur. In all those types of the trait Hb F is present in all erythrocytes in similar concentrations. The Swiss *type of hereditary persistence of Hb F* differs from the former traits by lower concentrations of foetal haemoglobin in heterozygotes (1–2.5%) and its unequal concentration in individual erythrocytes. The morphology of red blood cells in Swiss type of hereditary persistence of Hb F is normal, in contradistinction to thalassaemia minor in which similar levels of Hb F occur [3, 4].

In the case to be reported in this communication Swiss type hereditary persistence of Hb F has been diagnosed on the basis of family study. *In the course of an acquired haemolytic anaemia in this case* high Hb F concentrations (up to 23%) were found.

Case Report

The patient A. K., 32, housewife, was admitted to the Department of Internal Medicine in March 1968 because of anaemia and jaundice. The first symptoms of general weakness, pains of joints and jaundice occurred in November 1967. Viral hepatitis was diagnosed at that time, but was not confirmed following 3 weeks hospitalization. At the time of admission no enlargement of the spleen or liver was found. She did not report to have any haematological disorder before.

The results of determinations of total haemoglobin, Hb F, total serum bilirubin and reticulocytes are given in figure 1. The red blood cell mean corpuscular volume was 100 μm^3. No direct reacting bilirubin was found in the serum in the course of the study. The results of the direct and the indirect Coombs' tests were positive (6. 3. 68, direct Coombs test titre 1: 32). The osmotic fragility of erythrocytes was increased (6. 3. 68, 0.7–0.46% NaCl). The orthochromatic erythroblasts comprised 6% of nucleated cells in the peripheral blood (6. 3. 1968) and numerous spherocytes, macrocytes and megaloblasts were also present. Serum iron was 170 μg%. The $T\frac{1}{2}$ of ^{51}Cr labelled erythrocytes was 6.5 days and the index of activities above the spleen and above the liver was 3.6 (normal values 1.5–2.4, July 28–30, 1968)*. The activities of glucose-6-phosphate dehydrogenase and of the phosphoenolpyruvate kinase in erythrocytes were slightly elevated.

Autoimmune acquired haemolytic anaemia has been diagnosed on the basis of the results reported. Patient's condition has been improving during the treatment with prednisone (encorton, Polfa) and after the transfusions of red blood cells suspended in saline (direct Coombs test titre 4. 7. 68, 1: 32). A haemolytic crisis has occurred on August 23, 1968 (direct Coombs test titre 1: 64). Since the previous ^{51}Cr study has indicated that the spleen

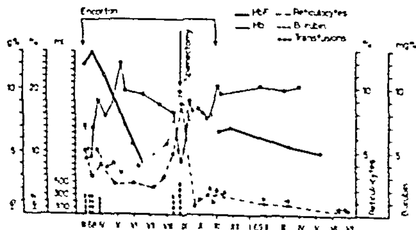


Fig. 1. Changes in the haemoglobin concentration, Hb F content, total serum bilirubin and percentage of reticulocytes in the patient's (A. K.) blood during the study.

* Results of this study were provided by Dr. M. [illegible] from the 2nd Department of Internal Medicine, Medical School, Poznań.

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Swiss Type Hereditary Persistence of Foetal Haemoglobin in a Case of Acquired Haemolytic Anaemia

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In the case to be reported in this communication Swiss type hereditary persistence of Hb F has been diagnosed on the basis of family study. In the course of an acquired haemolytic anaemia in this case high Hb F concentrations (up to 23%) were found.

In the haemolysates a fraction of haemoglobin with the electrophoretic mobility of Hb F has been found. The quantity of this fraction (21%) corresponded well to the concentration of the alkali resistant haemoglobin in this haemolysate (21. 3. 1968).

The changes in the Hb F level during the study are presented on figure 1. Lowering of the Hb F content coincided with the increase in total Hb content. As no transfusions were given April–June 1968 this coincidence suggests that lowering of Hb F level may be related to the reduced intensity of haemolysis.

The heterogeneous distribution of Hb F in the erythrocytes has been demonstrated (fig. 3b). Furthermore differences in Hb F content were found in erythrocytes of the patient different in relation to age and sedimentation properties (4. 3. 1969). Red blood cells suspensions in 0.9% NaCl (hematocrit 20%) were centrifuged in 10 cm long test tubes for 10 min at 600 g. The Hb F concentration was found to be 8% in the



Fig. 3. The [illegible] of Hb F [illegible] The H [illegible]
[illegible] Hb F [illegible] Hb F

was the main site of accelerated haemolysis, splenectomy was performed. A lung infarct occurred following this operation. As the thromboelastogram showed increase in the coagulability, anticoagulants (heparin and later phenhydren, Polfa) have been administered. After splenectomy lowering of bilirubin concentration and an increase in the total haemoglobin were noted (fig. 1). Direct Coombs test titre has been as follows: 20. 9. 68 1: 64, 24. 10. 68 1: 32, 4. 3. 69 1: 32.

The concentration of Hb F in haemolysates was determined with 2 methods: (1) alkali denaturation of cyanhaemoglobin [5]. Normal values in our conditions are up to 1% in adults, (2) electrophoresis on starch gel in the vertical system of SMITHIES [6] with continuous buffer tris-EDTA boric acid, pH 8.6 [7]. After separation of Hb A and Hb F gel was stained with bromophenol blue. Then the Hb-bound stain was eluted separately and its amounts were determined spectrophotometrically [8].

Histochemical staining of blood smears for Hb F in erythrocytes was made according to BETKE and KLEIHAUER [9].

Determinations of Hb A_2 were performed by starch block electrophoresis in barbital buffer 0.06 M, pH 8.6 [10]. The electrophoresis was carried out at 4°C with current 3 mA per cm² of the cross section of the block.

The test for autohaemolysis was done as described by YOUNG [11].

Activity of glucose-6-phosphate dehydrogenase was determined with the method of ZINKHAM [12] and that of phosphoenolopyruvate kinase with the BUCHER's method according to WIESMANN's modification [13].

The remaining laboratory investigations were carried out using standard methods.

Results

Increased autohaemolysis of the patients blood was noted at the admission (48 hours' incubation *in vitro*): defibrinated blood 20% (normal up to 2%), defibrinated blood with glucose 23% (normal up to 1%), defibrinated blood with ATP 15% (normal up to 1%).

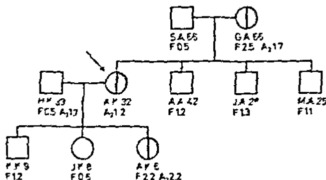


Fig. 2. Family tree. The concentrations of haemoglobin F and haemoglobin A_2 (in %) and the age of members of patient's family. The patient is indicated with arrow. Subjects suspected to have a Swiss type hereditary persistence of Hb F are designated as half-shaded circles.

In the haemolysates a fraction of haemoglobin with the electrophoretic mobility of Hb F has been found. The quantity of this fraction (21%) corresponded well to the concentration of the alkali resistant haemoglobin in this haemolysate (21. 3. 1968).

The changes in the Hb F level during the study are presented on figure 1. Lowering of the Hb F content coincided with the increase in total Hb content. As no transfusions were given April–June 1968, this coincidence suggests that lowering of Hb F level may be related to the reduced intensity of haemolysis.

The heterogenous distribution of Hb F in the erythrocytes has been demonstrated (fig. 3b). Furthermore differences in Hb F content were found in erythrocytes of the patient different in relation to age and sedimentation properties (4. 3. 1969). Red blood cells suspensions in 0.9% NaCl (hematocrit 20%) were centrifuged in 10 cm long test tubes for 10 min at 600 g. The Hb F concentration was found to be 8% in the



Fig. 3. The [illegible] of Hb F [illegible] The [illegible] (Hb F) [illegible]

top fraction of erythrocytes (reticulocyte count [illegible]), [illegible] in the middle fraction and [illegible] in the bottom fraction. The unseparated red blood cells contained [illegible] of Hb F (reticulocyte count [illegible]).

The Hb F concentration in red blood cells resistant to hemolysis in [illegible] NaCl (about [illegible] of cells lysed) has been [illegible] with [illegible] in whole blood (Table [illegible]). This however can be interpreted as the result of diminished osmotic resistance of red blood cells containing [illegible].

To investigate the possibility of the familial character of the increase in Hb F concentration in the patient, her parents, brothers and children have been studied (Fig. 2). All her relatives studied were in good health. Hb A_2 level and the red cell mean corpuscular volume were in normal range. The level of Hb F was however elevated in the mother (C.A.) and daughter (A.K.) of the subject. Heterogeneous distribution of Hb F in erythrocytes was demonstrated histochemically in these cases (Fig. 1).

Discussion

The elevated level of Hb F in the course of the acquired autoimmune hemolytic anaemia in the subject under study is interesting because of the prevailing Swiss type hereditary persistence of fetal haemoglobin. Family studies in Swiss type hereditary persistence of Hb F [illegible] have established the dominant character of this trait. High levels of Hb F in the blood of the mother and daughter of the patient support the diagnosis of Swiss type hereditary persistence of Hb F in the patient (Fig. 2).

The diagnosis of β-thalassemia in the investigated family can be excluded as no microcytosis and hypochromia have been found. The normal level of Hb A_2 provides an additional evidence against the diagnosis of β-thalassemia. Spherocytosis has been found in the patient, but not in the other members of the family. This finding is consistent with autoimmune, but not with the genetically determined dominant spherocytosis. The partial correction of autohaemolysis by addition of ATP cannot be explained by the [illegible] type [illegible] enzyme deficiency as the activity of this enzyme has been found to be normal.

Levels of Hb F ranging from [illegible] to [illegible], have been described in cases of acquired anaemias in adults [illegible]. Nevertheless, in studies in

patients with elevated Hb F levels in the course of aplastic anaemia no cases of the Swiss type hereditary persistence of Hb F have been found [16, 17].

While the homogenous distribution of Hb F in erythrocytes in cases with the increased Hb F level is a pathognomic symptom of some types of the hereditary persistence of Hb F, the heterogeneous distribution of this haemoglobin can be found in all remaining cases [18].

In the case investigated the greater concentration of Hb F in the bottom layer of red blood cells suggests that the life span of cells containing Hb F is longer than that of Hb A containing cells. A greater concentration of Hb F in the bottom layer of red blood cells has been already described in the heterozygotes of β-thalassaemia [19]. In this condition the longer life span of Hb F containing cells has been verified by the finding that the glycine 2-^{14}C-labelled Hb F remains longer in the circulation than the labelled Hb A [20]. This difference is believed to increase the compensatory effect of increased γ-chain synthesis in β-thalassaemia. In the patient studied the longer life span of the Hb F containing cells is interesting because of the different autoimmune mechanism of accelerated haemolysis. It might be speculated that longer life span of these cells in the patient can be related to presumptive differences in the antigenicity of Hb F containing erythrocytes as compared to Hb A containing cells.

If we consider the patient to have the Swiss type hereditary persistence of Hb F, the supposed differences in the red blood cells life span would explain the Hb F concentrations found in peripheral blood. If 2% of erythrocytes produced contained Hb F and had the normal life span, the shortening of the life span of the remaining erythrocytes to one fifth of the normal would cause the increase in the percentage of circulating Hb F containing cells to 10%. With normal compensatory capacity of the bone marrow, this would not cause anaemia [21]. Anaemia found in the patient at the beginning of the study corresponds to the shortening of the mean red blood cells life span to less than one tenth of the normal. Assuming a normal life span of Hb F containing cells, differences in the rates of haemolysis would explain the concentrations of 20% of Hb F in peripheral blood. The diminution of Hb F level coinciding with the reduced intensity of haemolysis (fig. 1, April–June, 1968) seems to confirm this interpretation.

A different mechanism of the increase in Hb F level in cases with accelerated erythropoesis has been proposed by Baglioni [22]. He

top fraction of erythrocytes (reticulocyte count 2.5%), 8.7% in the middle fraction and 14% in the bottom fraction. The unseparated red blood cells contained 11% of Hb F (reticulocyte count 1.0%).

The Hb F concentration in red blood cells resistant to haemolysis in 0.5% NaCl (about 50% of cells lysed) has been 18% (with 12% in whole blood, 21.5.1968). This however can be interpreted as the result of diminished osmotic resistance of red blood cells transfused 8–9 weeks earlier.

To investigate the possibilities of the familial character of the increase in Hb F concentration in the patient, her parents, brothers and children have been studied (fig. 2). All her relatives studied were in good health. Hb A_2 levels and the red cell mean corpuscular volume were in normal range. The level of Hb F was however elevated in the mother (G. A.) and daughter (A. K.) of the subject. Heterogenous distribution of Hb F in erythrocytes was demonstrated histochemically in these cases (fig. 3a).

Discussion

The elevated level of Hb F in the course of the acquired autoimmune haemolytic anaemia in the subject under study is interesting because of the coexisting Swiss type hereditary persistence of foetal haemoglobin. Family studies in Swiss type hereditary persistence of Hb F [3, 4] have established the dominant character of this trait. High levels of Hb F in the blood of the mother and daughter of the patient supports the diagnosis of Swiss type hereditary persistence of Hb F in the patient (fig. 2).

The diagnosis of $\beta\delta$ thalassaemia in the investigated family can be excluded as no microcytosis and hypochromia have been found. *The normal level of Hb A_2 provides an additional evidence against* the diagnosis of β thalassaemia. Spherocytosis has been found in the patient, but not in the other members of the family. This finding is consistent with symptomatic, but not with the genetically determined dominant spherocytosis. The partial correction of autohaemolysis by addition of ATP cannot be explained by the TANAKA type [14] pyruvate kinase deficiency as the activity of this enzyme has been found to be elevated.

Levels of Hb F ranging from 5 to 25% have been described in cases of acquired anaemias in adults [15–17]. Nevertheless, in families of

patients with elevated Hb F levels in the course of aplastic anaemia no cases of the Swiss type hereditary persistence of Hb F have been found [16, 17].

While the homogenous distribution of Hb F in erythrocytes in cases with the increased Hb F level is a pathognomic symptom of some types of the hereditary persistence of Hb F, the heterogeneous distribution of this haemoglobin can be found in all remaining cases [18].

In the case investigated the greater concentration of Hb F in the bottom layer of red blood cells suggests that the life span of cells containing Hb F is longer than that of Hb A containing cells. A greater concentration of Hb F in the bottom layer of red blood cells has been already described in the heterozygotes of β-thalassaemia [19]. In this condition the longer life span of Hb F containing cells has been verified by the finding that the glycine 2-^{14}C-labelled Hb F remains longer in the circulation than the labelled Hb A [20]. This difference is believed to increase the compensatory effect of increased γ-chain synthesis in β-thalassaemia. In the patient studied the longer life span of the Hb F containing cells is interesting because of the different autoimmune mechanism of accelerated haemolysis. It might be speculated that longer life span of these cells in the patient can be related to presumptive differences in the antigenicity of Hb F containing erythrocytes as compared to Hb A containing cells.

If we consider the patient to have the Swiss type hereditary persistence of Hb F, the supposed differences in the red blood cells life span would explain the Hb F concentrations found in peripheral blood. If 2% of erythrocytes produced contained Hb F and had the normal life span, the shortening of the life span of the remaining erythrocytes to one fifth of the normal would cause the increase in the percentage of circulating Hb F containing cells to 10%. With normal compensatory capacity of the bone marrow, this would not cause anaemia [21]. Anaemia found in the patient at the beginning of the study corresponds to the shortening of the mean red blood cells life span to less than one tenth of the normal. Assuming a normal life span of Hb F containing cells, differences in the rates of haemolysis would explain the concentrations of 20% of Hb F in peripheral blood. The diminution of Hb F level coinciding with the reduced intensity of haemolysis (fig. 1, April–June, 1968) seems to confirm this interpretation.

A different mechanism of the increase in Hb F level in cases with accelerated erythropoiesis has been proposed by Bacuosi [22]. He

supposed the greater concentration of Hb F in erythrocytes released from the bone marrow after fewer than normal number of divisions of progenitor cells

Further studies are necessary to see how often the increases of Hb F level in the course of accelerated haemolysis appear in patients with the Swiss type hereditary persistence of Hb F, and to estimate the difference in the life span of Hb A and of Hb F containing erythrocytes in these cases.

Acknowledgements We should like to thank Prof A HORST for suggestions concerning immune differences between the Hb F and the Hb A containing cells as well as to Prof K JASINSKI for initiation of the study

Summary

In a woman 32 years old an increase in foetal haemoglobin (Hb F) level (23-18%) has been found in the course of autoimmune haemolytic anaemia No hypochromia and no increase in Hb A_2 level have been found in the patient and in the members of her family The family investigation corroborate the diagnosis of the Swiss type hereditary persistence of Hb F in patient's mother, daughter and in the patient The higher concentration of Hb F in the bottom layer of erythrocytes is considered as evidence of accelerated haemolysis of Hb A containing cells as compared with Hb F containing cells This difference in red blood cells life span is discussed in relation to the observed Hb F levels and to the presumptive differences in the antigenicity between the Hb F and the Hb A containing cells

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Varia

International Society of Blood Transfusion

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(West Germany)

Morning session (9–11 a m) 10–12-min presentations Chairmen A J ZUCKERMAN [illegible]

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Britain), A J ZUCKERMAN (Great Britain)

Afternoon session (3 6 p m) 6–8-min short presentations Chairmen B S BLUMBERG (USA), J P. SOULIER (France) Speakers L I. BARKER (USA), H BERTHOLD (West Germany), F. DEINHARDT (USA), D J. GOCKE (USA), G A LOGRIPPO (USA), F O MACCALLUM (Great Britain), R H PURCELL (USA), G RITTNER (West Germany), M SEYFRIED (Poland), P TAYLOR (Great Britain)

General discussion between all the speakers

All applications for this symposium should be sent to Prof J P SOULIER, General Secretary of the International Society of Blood Transfusion, 6, rue Alexandre Cabanel, [illegible]

[illegible]

to attend this symposium) or pay a one day fee only for this special day (Tageskarte)

VI. Kongress der Weltfoderation für Hämophilie

Baden bei Wien, 25–27 Juli 1970

Veranstalter Österreichische Haemophilie-Gesellschaft unter Mitwirkung der Wiener Medizinischen Akademie für ärztliche Fortbildung

Tagungsort Kongresshaus, Baden bei Wien

Wissenschaftliche Themen 1 Neue Forschungsergebnisse auf dem Gebiet der Hämophilie 2 Standardisierung der Methoden für die Bestimmung des Hämophiliefaktors 3 Therapie der Hämophilie, a) Neue Faktor VIII- und Faktor IX-Konzentrate, b) Zahnpflege, c) Orthopädie und Unfälle, d) Diskussion über Nebenerscheinungen

In der paramedizinischen Sektion werden Diskussionen über drei Themen abgehalten 1 Die Schule und berufliche Probleme 2 Krankenversicherung 3 Rationalisierung der Therapie und Herabsetzung ihrer Kosten

Kongress Sprachen Deutsch, Englisch, Französisch, Spanisch

Teilnehmerbeiträge ÖS 750 für aktive Teilnehmer, ÖS 250 – für Begleitpersonen

Anfragen und Anmeldung Sekretariat c/o Wiener Medizinische Akademie, Stadiongasse 6–8, A 1010 Wien (Austria)

Acta haemat. 47: 193-209 (19[illegible])

Experimental Studies on Leukemia in Guinea Pigs[1]

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Leukemia and lymphosarcoma have long been observed to develop occasionally in middle-aged guinea pigs. The lymphoid tumors could be transmitted by cell graft, inducing disseminated lymphosarcomas and generalized leukemia in guinea pigs [6, 10, 11].

In 1954, CONGDON and LORENZ [1] observed several cases of leukemia developing spontaneously in guinea pigs bred at the National Cancer Institute in Bethesda, Md. Among a total of 303 guinea pigs observed, 10 animals developed leukemia, an incidence of 3.3%. Transplantation was accomplished in young guinea pigs of the same strain [1].

In subsequent studies dealing with experimental transmission of L2B and L2C leukemia, which originated in 'strain 2' guinea pigs, JUNGEBLUT and KODZA [4, 5] employed either young adult 'strain 2' guinea pigs or first generation hybrids. Both were consistently susceptible to inoculation of either L2B or L2C leukemia. Inoculation of cell suspensions prepared from leukemic spleens induced stem-cell leukemia after 2 to 3 weeks. Transmission by centrifuged, presumably cell-free extracts prepared from leukemic spleens was also reported [4]; the extracts were centrifuged 1 to 3 consecutive times at 5,500 to 10,000 r.p.m. The final supernate was inoculated, and induced leukemia after 15 to 21 days. Inoculation of plasma harvested from leukemic animals induced leukemia after 15 to 30 days. Attempts to transmit leukemia by filtered extracts were reported to have been successful in a few instances [4]; the disease developed in the inoculated animals

[1] Aided in part by grants from the Damon Runyon Memorial Fund, the American Cancer Society, and the Virginia and D. K. Ludwig Foundation.

after a short latency, not different from that observed following inoculation of blood plasma or of leukemic cell suspensions

However, in a similar study, carried out in another laboratory leukemia could not be transmitted in guinea pigs by leukemic extracts passed through either Seitz or Millipore filters [E M NADEL, personal communication to the author, 1967] In the same study transmission by centrifuged extracts was successful, blood plasma that had been centrifuged several times at low speed followed by high speed centrifugation at 35 000 rpm was also leukemogenic on inoculation tests

Experiments on Transmission of L2C Guinea Pig Leukemia Carried out in our Laboratory

We are very grateful to Dr C W JUNGEBLUT, Lenox Hill Hospital New York City, from whom we obtained on August 8 1968 a (Hartley × strain 2) F_1 hybrid guinea pig with fully developed (L2C strain) transplanted leukemia A cell suspension prepared aseptically from spleen, lymph nodes and from a leukemic tumor removed from this animal was inoculated subcutaneously into 2 young adult F_1 hybrid guinea pigs About 2 weeks [illegible] followed by [illegible]

Animals used 'Strain 2 guinea pigs were purchased from Horton s Laboratory Animals [illegible]

experiments The Hartley guinea pigs were purchased from Camm Research Institute, Wayne, N J Both 'strain 2 guinea pigs and F_1 hybrids were found to be consistently susceptible to induction, by cell-graft, of L2C leukemia Hartley guinea pigs are resistant

Induction of leukemia by cell-graft Within 2 to 3 weeks after subcutaneous inoculations of leukemic cell suspensions of 10 or 20% concentration into either 'strain 2' guinea pigs or F_1 hybrids, small, firm, subcutaneous tumors developed at the site of inoculation, increased in size gradually and were followed within a week to 10 days by the development of generalized leukemia One of the most prominent features of the induced leukemia was a consistent increase in the number of white cells in peripheral blood, with appearance of primitive stem cells and development of anemia, this striking change in peripheral blood morphology was first noticed several days after the development of subcutaneous tumors at the site of inoculation The number of white cells then increased gradually, reaching, usually within 5 to 10 days, in terminal phase of the disease, 300,000 to 500,000 WBC/mm^3, with predominance of primitive stem cells

Preparation of leukemic cell suspension. A guinea pig with advanced leukemia was sacrificed by ether inhalation. After the skin was shaved the abdominal cavity was exposed. Fragments of spleen, of the subcutaneous tumor which developed at the site of inoculation, of the mesenteric tumor, and in some instances also of a peripheral lymph node and of the liver were removed aseptically, weighed, cut with scissors, and ground by hand in a mortar, sterile physiological saline solution being added to obtain a cell suspension of either 10 or 20% concentration. The cell suspension was then passed through a sterile voile cloth and used immediately for inoculation. In some instances, when susceptible animals were not available for inoculation, the cell suspension was frozen in carbon dioxide dry ice at −70°C and used for inoculation one or several weeks later, occasionally after longer intervals.

Serial Passage by Subcutaneous Inoculations of Leukemic Cell Suspensions

Consecutive serial cell-graft passages were accomplished by subcutaneous inoculations of leukemic cell suspensions of 10 or 20% concentration into young adult guinea pigs. In each passage 2 or 3 guinea pigs were inoculated as soon as one of the inoculated animals developed generalized leukemia and the peripheral white blood count reached a high level.

Table I. Results of subcutaneous inoculation of leukemic cell suspensions into young adult strain 2 and F_1 hybrid guinea pigs

Strain	Leuk. cell conc. %	Dose ml	No. guinea pigs inoc.[a]	No. dev. leuk.	Leuk. inc. %
Strain 2	20	2	24	24	
	20	2	13	13	
	10	1	14	14	
Total			51	51	100
F_1 hybrids[b]	20	2	9	8[c]	
	20	1	3	2[c]	
	10	1	4	4	
Total			16	14	87

Summary: [illegible] tumor [illegible] each, 1 or 2 animals were inoculated. [illegible] cell suspensions were used immediately after preparation [illegible] had been kept at [illegible] °C for 9 to 15 [illegible]. Average [illegible] from inoculation [illegible] 14 days. Average [illegible] inoculation [illegible] terminal phase of disease [illegible] days.

[a] Approximately [illegible] old at time of inoculation.
[b] [illegible] strain 2 [illegible].
[c] One guinea pig [illegible] 31 days, another after 15 days.

Table II Titration experiments Induction of leukemia with serial dilutions of leukemic cell suspensions

Strain	Leuk cell conc	No guinea pigs inoc [1]	No dev leuk	Leuk inc %	Avg initial latency days[3]	Avg terminal latency days[3]
'Strain 2'	10^{-2}	5	5	100	15	21
	10^{-3}	9	9	100	16	24
	10^{-4}	5	5	100	25	48
F_1 hybrids[2]	10^{-3}	4	3[4]	75	16	21
	10^{-4}	2	2	100	22	42

[1] All inoculations subcutaneous, 1 ml each, into young adult guinea pigs, approximately 1 to 2 months old

[2] (Hartley ♀ × strain 2 ♂) F_1 hybrids

[3] Initial latency, from inoculation to appearance of tumors at site of inoculation Terminal latency, from inoculation to terminal phase of disease

[4] One still alive and well at 2½ months of age

the animal was sacrificed, and its spleen and leukemic tumors were used for the preparation of a cell suspension serving for the next passage (table I) Fifty-one 'strain 2' guinea pigs and 16 F_1 hybrids were inoculated All 51 'strain 2' guinea pigs and 14 out of 16 F_1 hybrids developed subcutaneous leukemic tumors at the site of inoculation after a latency varying in most instances from 10 to 15 days, followed a few days later by the development of generalized leukemia (table I)

The terminal phase of the disease was usually reached about 3 weeks after inoculation The disease was progressive in all instances There was no spontaneous recovery. All leukemic animals died, or were sacrificed when moribund, in terminal phases of the disease.

When higher dilutions of the leukemic cell suspensions were inoculated, the development of disease was delayed by one or two, or occasionally by three weeks (table II).

Dosage Induction of leukemia with serial dilutions of leukemic cell suspensions An attempt was made to determine the dose and concentration of leukemic cell suspensions necessary to induce leukemia, following subcutaneous inoculation into young adult 'strain 2' guinea pigs Cell suspensions of 10 or 20% concentration were uniformly leukemogenic, and induced a rapidly progressing disease after a short latency of about 2 weeks When 10^{-3} or 10^{-4} dilutions were inoculated subcutaneously, leukemia was also induced in all 'strain 2', and in 5 out of 6 F_1 hybrid



Fig. 7. [illegible] female guinea pig born to a Hartley female and a "strain 2" male was inoculated subcutaneously when 21 days old with [illegible] of a leukemic cell suspension, and developed a subcutaneous tumor at the site of inoculation, followed by a generalized leukemia (41,000 WBC/mm³). Sacrificed 19 days after inoculation. Note the very large spleen (S) and liver, and also enlarged lymph nodes (LN) in the axillary area and in the cervical region.

guinea pigs (table II), but the latency was prolonged. The induced disease did not differ from that resulting from inoculation of larger doses, except for the initial delay in the development of tumors and subsequent symptoms of generalized leukemia.

Inoculation of leukemic cell suspensions into young adult Hartley guinea pigs Two young adult Hartley guinea pigs were inoculated subcutaneously with large doses (2 5 ml) of a leukemic cell suspension of 20% concentration One was sacrificed after 80 days and another 190 days after inoculation Neither developed leukemia

Transmission by plasma. It is of interest that leukemia could readily be induced in young adult 'strain 2' or F_1 hybrid guinea pigs by subcutaneous inoculation of centrifuged, but not filtered, plasma

Blood mixed with a few drops of heparin was collected directly from the heart of anesthetized leukemic guinea pigs In most instances the blood was mixed with an equal volume of physiological saline solution The blood was centrifuged at 2,000 rpm for 10 min, plasma was then collected carefully, transferred to other tubes, and again centrifuged at 2,000 or 4,000 rpm for 5 or 10 min In two experiments, the second supernate was again centrifuged at either 2,000 or 4,000 rpm for 5 or 10 min In one experiment a fourth centrifugation was made at 9,500 rpm The final supernate was then inoculated subcutaneously

Out of 6 inoculated animals, 4 developed subcutaneous tumors at the site of inoculation, after a latency varying from 23 to 38 days, followed within a few days by generalized stem-cell leukemia However, when plasma was filtered, after a preliminary centrifugation, through Selas 02 filter candle, and then inoculated into 2 guinea pigs, no leukemia resulted

Attempt to transmit leukemia by centrifuged extracts. An attempt was made to transmit leukemia in guinea pigs by centrifuged, presumably cell-free extracts. In the first experiment, the leukemic cell suspension of 10% concentration was centrifuged at 3,000 rpm for 15 min. The supernate was centrifuged again at 9,500 rpm for 5 min; the second supernate was injected into a young adult 'strain 2' guinea pig. A local tumor developed at the site of inoculation after 31 days, followed shortly by generalized leukemia.

In 5 other experiments, the leukemic cell suspensions of 10 or 20% concentration were first centrifuged two or three consecutive times at 3,000 rpm for 15 min each. The second, or third supernate, respectively, was submitted to a final centrifugation at 9,500 rpm for 15 to 20 min. The final supernate was then inoculated subcutaneously (1 to 2 ml each), into 2 newborn, 3 less than 4-day-old, and 4 young adult guinea pigs. None of these animals has yet developed subcutaneous tumors or generalized leukemia They have been observed thus far for 2 to 3 months.

Attempt to Transmit Leukemia in Guinea Pigs by Filtered Extracts

An attempt was made to transmit leukemia in guinea pigs by filtered extracts. A total of 100 guinea pigs was inoculated. None of the inoculated animals has yet developed leukemia; they all are in good health and have been observed thus far for 3 to 16 months.

Leukemic cell suspensions of 10 or 20% concentration prepared from leukemic tumors and spleens in the usual manner were centrifuged at 3,000 rpm for 15 min; the supernate was centrifuged again at 9,000 rpm for 5 min. The second supernate was then passed through Selas 0[illegible] filter candles. Small groups of animals, one or two, in a few experiments up to 5 at a time, were inoculated in 39 experiments. Twenty-four filtered extracts were prepared, each from a different leukemic donor. Eleven filtrates were used immediately after preparation; all remaining filtrates were stored at −70 C for 1 to 174 days prior to their use for inoculation.

Seventy-nine newborn guinea pigs were inoculated subcutaneously with the filtered extracts (1 to 2 ml each) as follows: 15 strain 2, 36 F_1 hybrid, and 28 Hartley guinea pigs.

In addition, 21 young adult 1 to 2 month-old guinea pigs (19 strain 2 and 2 F_1 hybrids) were also inoculated subcutaneously (2 to 3 ml each; a few received 3 to 5 ml of the filtrates).

All inoculated animals are in good health at the time of this writing. They have been observed thus far for 3 to 16 months.

Pathologic Manifestations

Since the inoculations of leukemic cell suspensions were subcutaneous, the first manifestation was the induction of leukemic tumors at the site of inoculation. The subcutaneous tumors were at first circumscribed, moderately firm, with uneven edges; they soon infiltrated and adhered to the surrounding tissues, growing rapidly; within a few days they measured 1 or 2 cm in diameter, and became larger in terminal phases of the disease. Metastatic tumors developed in most instances, particularly in axillary and cervical lymph nodes, followed by a generalized leukemia.

The peripheral blood count had to be followed closely, since the animals usually reached the terminal phase of the disease only 5 to 10 days after they had developed subcutaneous tumors at the site of inoculation.

The tumors at the site of inoculation were [illegible] appeared [illegible] but not encapsulated. [illegible] the tumors were found to be made up of densely packed [illegible] tissues and [illegible] muscles. They consisted of large [illegible] packed [illegible] per high power field were noted [illegible]

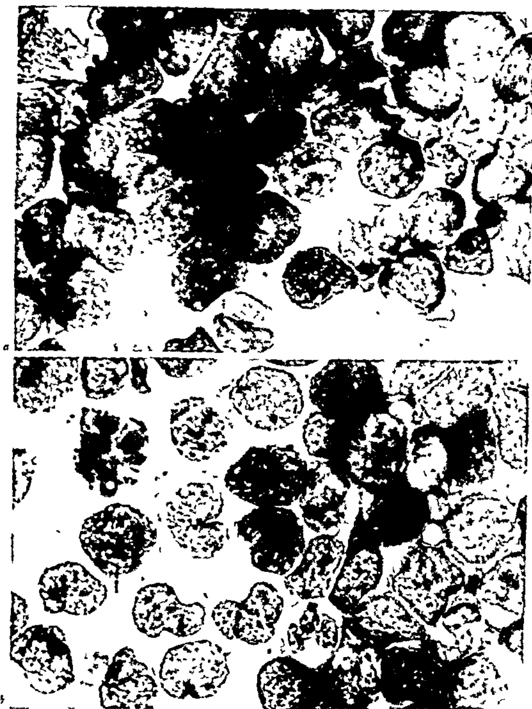


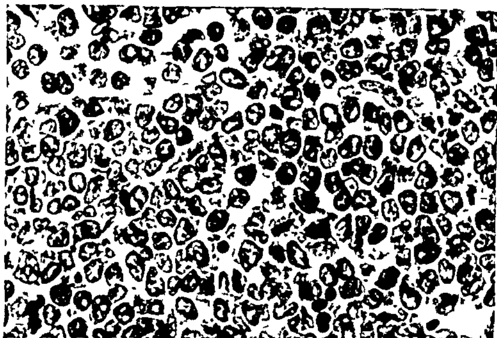
Fig 2 *a* Peripheral blood from a leukemic guinea pig in advanced phase of the disease (177,000 WBC/mm^3 with 63% of blast cells) Large numbers of undifferentiated stem cells are present (Wright stain, × 1,000) *b* Bone marrow from a guinea pig in advanced phase of disease (peripheral blood count 160 400 WBC/mm^3 with 90% of blast cells) Stem cells with large nuclei and nucleoli and occasional indented nuclei replaced the normal bone marrow element (Wright and Giemsa stain, × 1,000)

In the visceral leukemic infiltrations, the cells were found to grow in masses apparently without forming supporting stroma or capillaries (fig. 3a). However, at the site of inoculation, the tumors invading soft tissues and muscles had a fine stroma and many small capillaries, apparently growing with the tumors. Silver stains for reticulum (Gomori's silver methenamine) were done on representative sections of the various sites of leukemic tumor infiltrations. Reticulum fibers were present only in the tumors found at the site of inoculation but not in the visceral leukemic masses. The reticulum fibers in the tumors which developed at the site of inoculation were abundant and in places branched off into fine fibers which encircled individual cells. It is possible, but not likely, that the reticulum fibers are components of the soft tissues being replaced and not of the tumor. The pattern of growth of the inoculation site tumors would suggest that in soft tissues leukemia forms a lymphocytic or reticulum cell sarcoma.

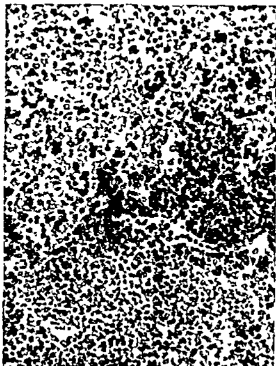
When the abdominal cavity of such animals was exposed, a prominent and striking feature was a considerable enlargement of spleen and liver; the spleen was frail, brittle and usually broke into fragments on attempt at removal. In advanced cases, the liver was very large, filling up a large part of the abdominal cavity. In practically all instances there was also a moderately large white, oblong, mesenteric tumor. The peripheral lymph nodes were frequently enlarged and replaced by leukemic tumors, particularly in the axillary area, and often also in the cervical region (fig. 1). Of particular interest was the fact that in no case did we observe thymic tumors.

On microscopic examination, there was involvement of virtually all organs, but particularly of liver (fig. 3b), and spleen, and to some extent also of peripheral lymph nodes, which showed widespread and extensive infiltration with leukemic cells. Other organs, such as kidneys, lungs, heart, and adrenals, showed a lesser degree of involvement. The infiltrating cells were similar to the stem cells of the peripheral blood. In the bone marrow and in tumorous tissues, the infiltrating cells resembled [illegible]. The cells were fairly uniform and some showed indentation of the nucleus, as seen in the peripheral blood. The [illegible] occasionally occurred around blood vessels, [illegible]. This was [illegible] by the frequent presence of tumor cells in the glomerular capillaries (fig. 3c). The [illegible].

Peripheral Blood and Bone Marrow. As leukemic animals approached the terminal phase of the disease, the peripheral blood count was usually very high (over 200,000 W.B.C./mm³) and consisted principally of large, primitive blast cells with large nuclei, 2 to 3 prominent nucleoli, scanty cytoplasm and an occasional indentation or infolding of the nucleus; the cytoplasm was light blue and often contained many vacuoles (fig. 2a). Numerous mitoses could be observed, particularly in some cases. Smudge cells were usually present. In addition to stem cells, there were small numbers of mature lymphocytes, polymorphonuclears, and less frequently metamyelocytes, basophils and eosinophils. Occasional "Kurloff bodies" were present.



a



b



c

Fig 3 *a* Leukemic tumor infiltrating the liver of a leukemic guinea pig in an advanced phase of the disease The cells are fairly uniform with large, occasionally indented nuclei prominent nucleoli and many mitoses (hematoxylin and eosin, × 570) *b* Same animal Low power photomicrograph of liver infiltrated with leukemic cells (hematoxylin and eosin, × 140) *c* Kidney glomeruli with leukemic cells filling the capillaries and afferent arteriole (Periodic-acid Schiff, × 220)

The so-called Kurloff bodies [9], peculiar to guinea pig blood, are found occasionally in the blood cells of both normal and leukemic guinea pigs. These are cytoplasmic inclusion bodies found in cells of the lymphocytic series. On blood smears stained routinely with Wright stain, they appear red and smooth or as vacuoles with dark red granules.

In some cases, the peripheral white cell count was in the 40,000 to 50,000 WBC/mm³ range and in these instances there were, in addition to the large number of blasts, also significant numbers of polymorphonuclears, as well as lymphocytes and occasionally also metamyelocytes. There was also a gradual development of anemia.

In all advanced cases of leukemia an extensive replacement of normal bone marrow population by cells resembling stem cells seen in the peripheral blood could be observed (fig. 2b).

The following is a random sample of hematologic studies performed on 15 leukemic guinea pigs in terminal phases of the disease. All showed a marked elevation in peripheral white blood cell counts, which ranged from 10[illegible] 000 to 518 000/mm³, with an average of 242 000/mm³. Blood smears showed a large percentage of [illegible] cells or blasts, ranging from 51 to 9[illegible]%, with an average of 8[illegible]%. (The stem cells were negative for peroxidase reaction.) The percentage of mature granulocytes was decreased to an average of 9%, and that of mature normal lymphocytes to an average of only [illegible]%. The number of platelets also appeared to be decreased.

The leukemic animals presented a marked anemia with hemoglobin values ranging from 4.4 to 10.2 g per 100 ml of whole blood. There was a moderate anisocytosis of red blood cells with presence of poikilocytes and crenated red blood cells, some diffuse and punctate basophilia, and an occasional nucleated red blood cell.

Electron Microscopic Studies[1]

Examination of ultrathin sections prepared from organs of 14 leukemic guinea pigs revealed in the cisternae of the endoplasmic reticulum in the leukemic cells the presence of doughnut-like virus particles; these particles had two concentric membranes, relatively electron lucent centers, and measured about 90 to 100 mμ in diameter; they appeared to be formed by budding from the membranes of the endoplasmic reticulum into the cisternae (fig. 4). In addition, mature spherical particles about 103 mμ in overall diameter, with thick rough outer envelopes and centrally located electron-dense nucleoids, were found in intercellular spaces; the average diameter of the nucleoids was about 50 mμ (fig. 5).

[1] In cooperation with [illegible]. A more detailed description of the electron microscopic observations in guinea pig leukemia will be published in a separate paper.

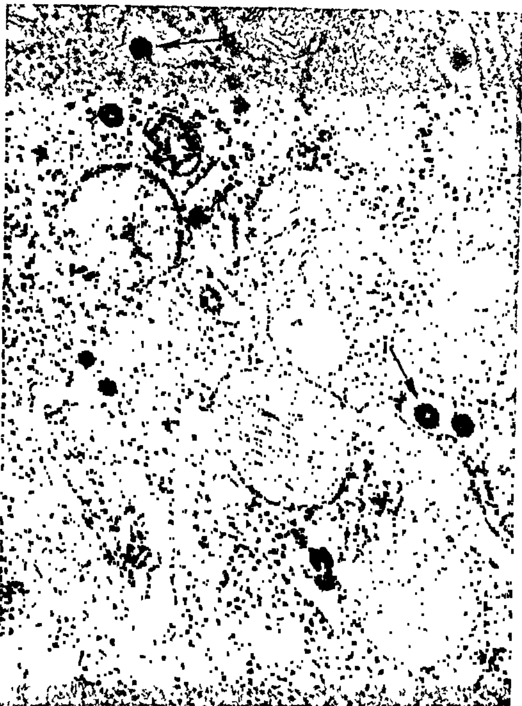


Fig 4 Ultrathin section of a fragment of a leukemic cell infiltrating the lungs in a leukemic 'strain 2' guinea pig This animal was inoculated with leukemic plasma, developed a generalized stem-cell leukemia (183,500 WBC/mm³) and was sacrificed, when moribund, 46 days after inoculation Within the cisternae of the endoplasmic reticulum are budding (b) and immature (i) leukemic virus particles (× 68,000)

Virus particles observed in our studies of guinea pig leukemia were essentially similar to those previously described by OPLER [8] and also by NADEL *et al.* [7].

The doughnut like particles and particles with nucleoids were found in organs of all 14 animals examined; they were observed in a variety of tissues, such as subcutaneous and visceral leukemic tumors, spleen, lymph nodes, and bone marrow, as well as in leukemic cells infiltrating the liver, ovaries, uterus and lungs.

In a control study, no virus particles were found either in the endoplasmic reticulum or in the intercellular spaces of tissue fragments of spleen, liver, lymph nodes, or bone marrow from 9 normal, healthy 'strain 2' guinea pigs examined [3].

The virus particles found in organs of leukemic guinea pigs were different from those observed in mouse, rat, or cat leukemia [2, 3]. They were less numerous and were located mostly in the endoplasmic reticulum; we have never observed these particles budding from the outer cell membranes; they had frequently a slightly smaller overall diameter, and smaller, more dense and more homogeneous nucleoids than virus particles observed in leukemic mice, rats, and cats. There was much less variation in the overall size of the virus particles in guinea pig leukemia as compared with particles observed in mouse leukemia. Furthermore, virus particles observed in guinea pig leukemia had a thick, granular, rather fuzzy outer coat.

The nucleoids of the mature mouse, rat, and cat leukemia virus particles vary in size, shape and density, whereas the nucleoids of the mature guinea pig virus particles are more regular in size, spherical shape and density, and vary much less in diameter.

In the mouse, rat and cat, immature leukemic virus particles form by budding from the outer cell membranes and from vacuolar membranes of megakaryocytes. In the guinea pig, on the other hand, immature particles form by budding from membranes of endoplasmic reticulum. Consequently, immature and also mature mouse, rat and cat leukemia virus particles are usually located in the intercellular spaces, whereas most of the guinea pig virus particles appear within cisternae of the endoplasmic reticulum, and only a few can be found in intercellular spaces.

In general, mouse, rat and cat leukemia virus particles were present in larger numbers in the organs of leukemic animals studied than guinea pig leukemia virus particles.



Fig. 5. Mature virus particles (m) in intercellular spaces from areas infiltrated with leukemic cells. *a* Fragment of an ovary, and *b* fragment of lungs, from a 'strain 2' guinea pig with generalized stem-cell leukemia. Same animal as in figure 4 (*a* × 122,000, *b* × 116,000).

Discussion

The L2C strain of guinea pig leukemia could be transmitted consistently, by cell graft, in "strain 2" or in F_1 hybrid guinea pigs, inducing a rapidly progressing stem-cell leukemia in the inoculated animals. No spontaneous regression of the induced disease was observed in any case among the 65 guinea pigs which developed leukemia as a result of subcutaneous inoculation of leukemic cell suspensions of 10 or 20% concentration.

Transmission by plasma was also accomplished; however, when the plasma was filtered and then used for inoculation, no leukemia developed in the inoculated animals.

Transmission by a centrifuged extract succeeded in one experiment in which only two consecutive centrifugations were made. In this animal a local tumor developed at the site of subcutaneous inoculation, followed later by generalized leukemia. No leukemia has been induced thus far with extracts that had been submitted to 3 or 4 consecutive centrifugations. However, guinea pigs inoculated with such extracts are relatively young, and are still being observed. Should these preliminary results be corroborated by further observations and should the remaining animals survive, free from leukemia, it would then appear that one or two consecutive centrifugations may not be sufficient to sediment all cells or cell fragments. The fact that a leukemic tumor developed in the only positive animal at the site of inoculation would be consistent with such an assumption.

Different experimental conditions may be valid for transmission of leukemia with plasma, because of the viscosity of such extracts. Even several successive centrifugations did not prevent plasma extracts from inducing leukemia on inoculation tests. It is possible that a few cells or cell fragments may remain in plasma extracts even after several centrifugations inducing again local tumors at the site of inoculation followed later by the development of generalized leukemia. No leukemia, however, could be transmitted thus far with a filtered plasma extract.

Transmission by filtered tumor and spleen extracts has not yet succeeded in our laboratory. However, our experiments are still in progress.

The apparent difficulty in transmitting leukemia in guinea pigs by filtered extracts has been stressed in previous studies. Although Jungeblut

BLUT and KODZA [4] reported transmission of guinea pig leukemia by filtered extracts, certain reservations must be raised in reference to these experiments; the latency was very short, about 3 weeks. The narrow and specific strain susceptibility to inoculation with either leukemic cell suspensions, centrifuged extracts, or plasma, is also significant; only 'strain 2' or F_1 hybrid guinea pigs were susceptible in our experiments as well as in those previously reported [1, 4, 5]. Short latency and a narrow strain susceptibility usually refer to transmission by cell-graft of tumors or leukemia, and would be rather unusual in experiments dealing with the induction of leukemia, or tumors, by a cell-free agent.

The presence of spherical virus-like particles with characteristic granular outer coats in organs of leukemic animals was striking and could be readily revealed on electron microscopic examination. These particles could be differentiated from virus particles observed in leukemic mice, rats, cats and chickens.

Summary

The L2C leukemia strain is consistently transmissible by cell graft in 'strain 2' or F_1 hybrid guinea pigs The inoculated animals developed tumors at the site of inoculation, followed by a generalized leukemia, the thymus was not affected Practically all animals developed in terminal phases of the disease very high white blood cell counts with predominance of stem cells

Transmission by centrifuged extracts was successful only in one experiment in which 2 successive centrifugations were employed, but not when the extracts were submitted to 3 or 4 successive centrifugations Transmission by plasma was accomplished, but not with plasma filtrate Seventy nine newborn and 21 young adult guinea pigs were inoculated with filtered spleen and tumor extracts, but none has thus far developed leukemia

Electron microscopic studies revealed consistent presence in the leukemic cells of spherical particles approximately 100 $m\mu$ in diameter, having a fuzzy, granular outer coat, some of them had electron-dense, centrally located nucleoids

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Wirkung von L-Asparaginase aus *Escherichia coli* auf die Stimulation von Lymphozytenkulturen[1]

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Stimulation von Lymphozyten *in vitro* wurde erstmals 1959 von Hungerford *et al.* und 1960 von Nowell als Folge der Einwirkung eines Phytohämagglutinins aus Phaseolus vulgaris (PHA) beschrieben [22, 30]. Dabei entstehen aus den Lymphozyten nach anfänglicher Steigerung der Protein- und RNS-Synthese die sogenannten Blasten, die eine hohe DNS-Syntheserate und Zellteilungsfrequenz aufweisen [8, 12, 27, 51]. Ausser dem PHA ist seither eine Anzahl von Stimulationsmechanismen gefunden und mehrfach zusammenfassend dargestellt worden [4, 25, 29, 35, 36, 46].

Kürzlich wurde gezeigt, dass das zytostatisch wirksame Enzym L-Asparaginase aus *E. coli* [neuere deutschsprachige Übersichten bei 6, 18, 34, 47] die PHA-bedingte Stimulation der Lymphozyten des Blutes Gesunder zu hemmen vermag [2, 24]. Da die Wirkungsweise des Enzymes im System der Lymphozytenkultur noch ungeklärt ist, erscheint es von Interesse, zu untersuchen, ob auch andere Stimulationsmechanismen durch L-Asparaginase in gleicher Weise beeinflusst werden können. In der vorliegenden Arbeit wurden Lymphozytenkulturen mit verschiedenen Antigenen [15, 40], Staphylokokken-Kultur-Filtrat [26], Streptolysin O [21], Pokeweed Mitogen [11, 16] und Quecksilber-II-chlorid [48] stimuliert und in jedem Fall der Einfluss von L-Asparaginase aus *Escherichia coli* untersucht.

[1] Wir bedanken uns bei Dr. Jeso von den Behringwerken Marburg für die Überlassung der L-Asparaginase und bei Dr. Kerster, Bakteriol. Untersuchungsstelle des Inst. für Hyg. und Med. Mikrobiol. der FU (Direktor Prof. Dr. B. Schmidt) im Klinikum Steglitz für den zur Herstellung des Staphylokokkenfiltrates verwendeten Stamm.

Gewebekulturmethoden

Lymphozytenkulturen wurden in folgender Weise unter Beachtung steriler Kautelen gewonnen. Heparinisiertes (20 IE/ml) Venenblut gesunder Freiwilliger wurde mit Macrodex 6% (Knoll) im Verhältnis 1:10 vermischt und nach 45 min mit der Sedimentation (Temp. 37 °C bei einem Neigungswinkel von 45 Grad) der leukozytenreiche Plasmaüberstand mit Pasteurpipetten vorsichtig entfernt. Die zur Kultivierung verwandten Zentrifugengläser (mit Silikonstopfen dicht verschlossen) enthielten jeweils 5 ml Kulturansatz, der aus Zellsuspension (4–6 Mill. weißen Zellen pro ml), Eigenserum und Medium TC 199 (Difco) im Verhältnis 1:1:4 hergestellt worden war. Die Endkonzentration der Antibiotika Penicillin und Streptomycin betrug 100 IE/ml. Angereicherte Lymphozytenfraktionen verwendeten wir bei diesen Versuchen nicht, da in letzter Zeit mehrfach gezeigt worden war, dass gerade die Anwesenheit von mononukleären Makrophagen in der Kultur in vielen Fällen für eine optimale Stimulation notwendig ist [17, 33].

Die stimulierenden Agentien und gegebenenfalls L-Asparaginase wurden den Kulturen zu Versuchsbeginn zugesetzt und diese dann über den jeweils notwendigen Zeitraum bei 37 °C in einem unbegasten Brutschrank inkubiert. Zu Versuchsende wurden nach Zentrifugation (ca. 700 rpm 10 min) von den Sedimenten Ausstriche angefertigt und in üblicher Weise nach Pappenheim gefärbt. Von jedem Ausstrich wurden zur Bestimmung des Prozentsatzes blastenartiger Zellen 1000 intakte lymphozytäre Zellen ausgezählt, wobei die Unterscheidung von Blasten und Makrophagen bei nahezu jeder Zelle auf Grund morphologischer Kriterien [9] allein möglich war und die sogenannten Zwischenformen nicht als Blasten gewertet wurden. Spontane Transformation zu Blasten war bei unbehandelten Kontrollen in unserem System sehr selten (unter 0,0 %). Hierin stimmen unsere Versuche mit den Ergebnissen von Carron [11] weitgehend überein.

Die Trypanblauversuche wurden nach der von Tenk *et al.* [[illegible]] angegebenen Modifikation durchgeführt.

Materialien

Die folgenden Substanzen wurden den Kulturen in Aliquots von 0,1 ml/Ansatz zugefügt; notwendige Verdünnungen erfolgten mit 0,9%iger Kochsalzlösung; angegeben sind die jeweiligen Endkonzentrationen: Phytohämagglutinin P (Difco) 2 µl/ml; Pokeweed Mitogen (Grand Island Biological Company) [illegible] µl/ml; Quecksilberchlorid p.a. (Merck) [illegible] µl/ml; Alt-Tuberkulin 2 µl/ml; Tetanol 2 µl/ml; Vaccinia-Antigen 20 µl/ml; L-Streptolysin O 2 E/ml; alle zuletzt genannten Substanzen von den Behringwerken. Die L-Asparaginase wurde freundlicherweise von den Behringwerken Marburg zur Verfügung gestellt und für die Versuche mit 0,9%iger NaCl so verdünnt, dass die Endkonzentrationen zwischen 0,5 und 10 E betrugen. [illegible] wurde nach der von [illegible] *et al.* beschriebenen Methode bereitet [[illegible]] und in Konzentrationen von 0,1 ml bis 1,0 ml pro Kulturansatz verwendet. Zusätzlich kam [illegible] (Behringwerke) in einer Konzentration von 0,0 [illegible] E/ml zur Anwendung.

Ergebnisse

Stimulation mit Antigenen. In einer früheren Versuchsserie (unveröffentlicht) hatten wir bei 20 gesunden Blutspendern die Reaktion der Blutlymphozyten in Kultur auf verschiedene Antigene untersucht. Es

handelte sich dabei um Alt-Tuberkulin, Tetanus Toxoid, Vaccinia-Antigen und Streptolysin O. Bei einer durchschnittlichen spontanen Transformationsrate von unter 0,05% wurden diejenigen Kulturen als positiv betrachtet, die mindestens 1% Blasten bei Auswertung von 1000 intakten lymphozytären Zellen nach einem 6tägigen Kulturintervall aufwiesen.

Zur Prüfung des Einflusses der L-Asparaginase auf die Antigen-induzierte Stimulation wurde bei den jetzigen *Experimenten die* Versuchsanordnung so gewählt, dass im Falle jedes einzelnen Antigens mindestens 5 verschiedene Versuche mit 5 verschiedenen Blutspendern durchgeführt wurden. Der Prozentsatz Blasten in den lediglich mit Antigen behandelten *Kontrollen aus diesen Versuchen ist in Tabelle I* dargestellt. Bei jedem Versuch wurde gleichzeitig eine Kontrolle mitgeführt, *die nur L-Asparaginase erhielt*. Die daraus gewonnenen Präparate erschienen sowohl nach morphologischen Kriterien wie auch betreff der Trypanblauversuche durch das Enzym völlig unbeeinflusst zu sein.

Wurde in diesen Versuchen L-Asparaginase aus *E. coli* zusammen mit dem Antigen zu Versuchsbeginn den Kulturen zugesetzt, so konnte damit in jedem Fall die Blastenentstehung vollständig verhindert werden, d. h. bei Untersuchung der gefärbten Ausstriche nach Beendigung der Kulturen waren keinerlei Blasten nachzuweisen, obwohl die typischen kleinen Lymphozyten auf den Ausstrichen in grosser Zahl morphologisch intakt erschienen und auch bei Untersuchungen mit Trypanblau der Prozentsatz gefärbter Zellen sich nicht wesentlich von den Kontrollen unterschied.

Andere Stimulationsmechanismen. Ausser den Antigenen verwendeten wir noch folgende weitere Substanzen zur Stimulation von Lympho-

Tabelle I. Prozentsatz sogenannter Blasten in antigenstimulierten Lymphozytenkulturen nach 6 Tagen mit und ohne Zusatz von L-Asparaginase aus *E. coli* (0,5 bis 1,0 IE/ml). Werte aus jeweils mindestens 5 Versuchen mit 5 verschiedenen *Blutspendern*

	L-Asparaginase	
	ohne	mit
Alt-Tuberkulin	2,30	0
Tetanus Toxoid	2,26	0
Streptolysin O	4,25	0
Vaccinia-Antigen	1,[illegible]9	0

Tabelle II. Prozentsatz sogenannter Blasten in stimulierten Lymphozytenkulturen mit und ohne Zusatz von L-Asparaginase aus *E. coli* (0,5–1,0 IE/ml). Werte aus jeweils mindestens 10 Versuchen mit 10 verschiedenen Blutspendern

	PHA[1]	PWM[2]	$HgCl_2$	SF[3]
Ohne Asparaginase	60–80	30–50	5–33	2–35
Mit Asparaginase	0	0	0	0

[1] Phytohämagglutinin aus Phaseolus vulgaris
[2] Pokeweed Mitogen
[3] Staphylokokken-Filtrat

zytenkulturen: Phytohämagglutinin P aus *Phaseolus vulgaris* (PHA), Pokeweed Mitogen (PWM) (Kulturdauer jeweils 3 Tage) sowie Staphylokokken-Filtrat (SF), Staphylolysin und Quecksilber-II-chlorid (Sublimat) bei 4tägigem Kulturintervall. Untersucht wurden jeweils mindestens 10 verschiedene Kulturansätze von 10 verschiedenen Blutspendern.

Die spontane Transformationsrate lag auch in den Kontrollen dieser Versuche unter 0,05%, lediglich mit L-Asparaginase behandelte Kulturen erschienen entsprechend den obigen Versuchen völlig unbeeinflusst. Der Prozentsatz stimulierter Zellen in den entsprechenden Versuchen ist in Tabelle II dargestellt. Wir finden dabei im Falle des PHA ziemlich konstant eine grosse Zahl Blastenzellen (60–80%), bei PWM waren es deutlich weniger (30–50%), die Stimulation war jedoch in den einzelnen Versuchen ebenfalls recht einheitlich. Im Gegensatz dazu standen die anderen Stimulationsmechanismen, bei denen erhebliche Unterschiede zwischen den einzelnen Blutspendern bestanden. Wir fanden bei Quecksilber-II-chlorid 5–33% Blasten und bei Staphylokokken-Filtrat 2–35%. Ein wesentlicher Unterschied zwischen Staphylokokken-Kultur-Filtrat und Staphylolysin, das vorwiegend Staphylokokken-Hämolysine enthält, bestand nicht.

Die Beeinflussung der Blastenentwicklung durch L-Asparaginase aus *E. coli* war bei allen diesen Stimulationsmechanismen gleich und entsprach den obigen Versuchen mit Antigenen. Die Stimulation liess sich durch gleichzeitige Gabe von Stimulans und L-Asparaginase bei Versuchsbeginn vollständig verhindern, so dass die bei Beendigung der Versuche angefertigten Ausstriche in allen Fällen lediglich unstimulierte Lymphozyten neben Makrophagen, [illegible] und degenerierenden polymorphkernigen aufwiesen. Dabei erschienen die

Lymphozyten nach morphologischen Kriterien und bei Anwendung der Trypanblaumethode als ungeschädigte lebende Zellen

Diskussion

Von vielen Untersuchern wird zwischen «spezifischer» und «unspezifischer» Stimulation von Lymphozyten *in vitro* unterschieden. Zur Begründung dient die Tatsache, dass Antigene nur bei vorheriger Sensibilisierung einen relativ kleinen Prozentsatz der Lymphozyten nach etwa 6tägigem Kulturintervall stimulieren, während andere Substanzen (am besten untersucht ist das PHA) einen weitaus grösseren Anteil der Zellen aller Gesunden nach kürzerer Kulturdauer zu sogenannten Blasten transformieren. In beiden Fällen werden in den in unbehandelten Kulturen inaktiven kleinen Lymphozyten ganz entscheidende morphologische und biochemische Veränderungen induziert (u.a. DNS-Synthese und Mitosen), deren Ursache jedoch weder bei den «spezifischen» noch bei den «unspezifischen» Mechanismen bekannt ist. Vor allem ist es nicht entschieden, ob der Stimulationsvorgang in beiden Fällen der gleiche ist, oder ob dabei wesentliche Unterschiede bestehen. Es erschien deshalb von Interesse zu untersuchen, ob die beschriebene Hemmung der PHA-Wirkung auf Lymphozytenkulturen durch L-Asparaginase [2, 24] auch bei verschiedenen anderen Stimulationsmechanismen nachweisbar ist.

Unsere Experimente ergaben, dass die Stimulationswirkung sämtlicher untersuchten Antigene durch gleichzeitige Gabe des Enzymes zu Versuchsbeginn verhindert werden konnte, d.h. Blasten waren in den nach 6 Tagen angefertigten Ausstrichen nicht nachweisbar. Das gleiche galt bei unterschiedlichem Kulturintervall für die Versuche mit Pokeweed-Mitogen, Staphylokokkenfiltrat und Quecksilber-II-chlorid. In allen Fällen erwies sich die L-Asparaginase als ausserordentlich wirksam in der Unterdrückung des Transformationsvorganges und wir haben in allen beschriebenen Experimenten tatsächlich keine einzige Blastenzelle nach Gabe von L-Asparaginase aus *E. coli* gefunden. Es bestand also hierin kein Unterschied zwischen «spezifischen» und «unspezifischen» Stimulationsmechanismen.

L-Asparaginase ist in der verwendeten Konzentration weitgehend atoxisch für normale unbehandelte Lymphozyten in Kultur [49, eigene Beobachtungen] und wir halten es auf Grund der Trypanblauver-

suche für unwahrscheinlich, dass im Falle der stimulierten Kulturen lediglich die Blasten oder deren Vorläufer als Folge der Enzymbehandlung selektiv vernichtet werden. Wir nehmen dagegen an, dass die Stoffwechselvorgänge, die zur Blastenbildung und Mitose führen, an irgendeiner Stelle so beeinflusst werden, dass die Transformation unterbleibt. Es ist möglich, dass es sich dabei nicht nur um eine Folge der Spaltung von exogenem Asparagin handelt, sondern dass für L-Asparaginase auch ein spezifischer intrazellulärer Angriffspunkt gegeben ist. Welcher Art die Stoffwechselvorgänge sind, die den Angriffspunkt des Enzymes darstellen, ist auf Grund der bisherigen rein morphologischen Untersuchungen nicht näher zu definieren. In diesem Zusammenhang konnte es jedoch von Interesse sein, dass bei Gabe der L-Asparaginase 12 und 24 Stunden nach Phytohämagglutinin noch eine Hemmung der Blastenbildung nachweisbar ist [24] und dass die Asparaginasewirkung auf PHA-Kulturen zu Versuchsende nicht nachweisbar ist, wenn die Zellen 12 Stunden nach Gabe von PHA und Enzym gewaschen werden und neues Medium erhalten (unveröffentlichte Beobachtungen).

Die anderen in der Literatur beschriebenen Agentien mit der Fähigkeit, die Transformation der Lymphozyten *in vitro* zu hemmen, sind recht vielfältiger Art. Es handelt sich dabei u. a. um Hormone [31, 32], Stoffwechselinhibitoren [23, 33, 41, 42, 44], Zytostatika [3, 20], Makromoleküle [5, 13, 37], Viren [21, 50, 53], Extrakte aus Mykoplasmen [14], Ouabain, Adamantinhydrochlorid und Natriumsalizylat [39, 43, 45]. Sämtliche dieser Möglichkeiten sind von grossem Interesse, da sie u. U. indirekten Aufschluss über den Stimulationsmechanismus der Lymphozyten erbringen können. Ein Enzym wie L-Asparaginase mit einer chemisch relativ einfach zu definierenden Wirkungsweise erscheint aus dem gleichen Grund besonders geeignet, weitere Untersuchungen an stimulierten Lymphozytenkulturen durchzuführen. Die Wirksamkeit der L-Asparaginase in diesem System ist neben den Befunden an regenerierender Rattenleber, an Kaninchen-Embryonen und Knochenmarkskulturen [1, 7, 19], ein zusätzlicher Hinweis dafür, dass dieses Enzym nicht nur spezifisch Tumorzellen beeinflusst, sondern auch andere schnell proliferierende Gewebe.

Zusammenfassung

[illegible]

hämagglutinin P, Pokeweed Mitogen, Quecksilber II-chlorid und Staphylokokken Kultur-Filtrat stimuliert In allen Fällen konnte die Entstehung von Blasten und Mitosen vollständig verhindert werden, wenn L Asparaginase aus *Escherichia coli* zusammen mit dem stimulierenden Agens zu Versuchsbeginn gegeben wurde Hierbei bestand kein Unterschied zwischen «spezifischen» und «unspezifischen» Stimulationsmechanismen

Summary

Normal peripheral lymphocyte cultures were stimulated with antigens (old tuberculin tetanus toxoid, vaccinia antigen and streptolysin O), as well as with Phytohaemagglutinin of phaseolus vulgaris, Pokeweed mitogen, staphylococcal filtrate and mercuric chloride In any case the development of blasts and mitosis in culture could be prevented completely when L asparaginase from *Escherichia coli* was added together with the stimulant at the beginning of the culture There was no difference between the so called specific and nonspecific stimuli

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Cytochemical Studies of Acute Leukemias

E. A. Leinonen

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In combining cytochemical and morphological analysis, an attempt was made to correlate alterations in cellular structure with differences in chemical composition. As recent evidence has indicated that the major defect in the acute leukemias is the impairment of cellular differentiation and maturation [5, 7], studies to obtain cytochemical profiles of the primitive cells were undertaken.

Materials and Methods

Blood and bone marrow films were obtained from 71 patients with acute leukemias: 29 with acute lymphocytic leukemia (ALL), 23 with acute myelomonocytic and acute monocytic leukemia (AML), 19 with acute granulocytic leukemia (AGL), including 5 patients in the acute phase of chronic granulocytic leukemia.

Controls consisted of blood and bone marrow films of 16 healthy adults and 3 patients with nonhematological diseases.

Six cytochemical reactions were used: periodic acid Schiff, Sudan black B, acid phosphatase, alkaline phosphatase, nonspecific esterase, and phosphorylase. The methods employed for the reactions were: [illegible] and [illegible] [10] for lipids; [illegible] and Barka [3] for acid phosphatase; [illegible] [1] for alkaline phosphatase; Wachstein and Wolf [9] for nonspecific esterase; Takeuchi and Kuriaki [7] for phosphorylase, and periodic acid Schiff reaction for glycogen and glycoprotein. Wright's stain was employed for morphological evaluation.

A semiquantitative scoring method was used for estimating the intensity of the cytochemical enzyme activity. The range of individual cells was from colorless to intense (0–4+). To obtain a total score, 100 cells of each type were counted for each reaction with 400 as the highest possible mean score.

Results

PAS reaction in lymphoblasts of ALL exhibits a moderate to intense reactivity (+ + +), monoblasts of AML weak to

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Six cytochemical reactions were used periodic acid Schiff, Sudan black B, acid phosphatase alkaline phosphatase nonspecific esterase and phosphorylase The methods employed for the reactions were Sheehan and Storey [16] for lipids Goldberg and Barka [9] for acid phosphatase Ackerman [1] for alkaline phosphatase Wachstein and Wolf [20] for nonspecific esterase Eränkö and Palkama [6] for phosphorylase, and periodic acid Schiff reaction for glycogen and glycoprotein Wright s stain was employed for morphological evaluation

A semiquantitative scoring method was used for estimating the intensity of the cytochemical enzyme activity The rating of individual cells was from colorless to intense (0–4+) To obtain a total score 100 cells of each type were counted for each reaction with 400 as the highest possible mean score

Results

PAS reaction in lymphoblasts of ALL exhibits a moderate to intense reactivity (++–+++), monoblasts of AML weak to

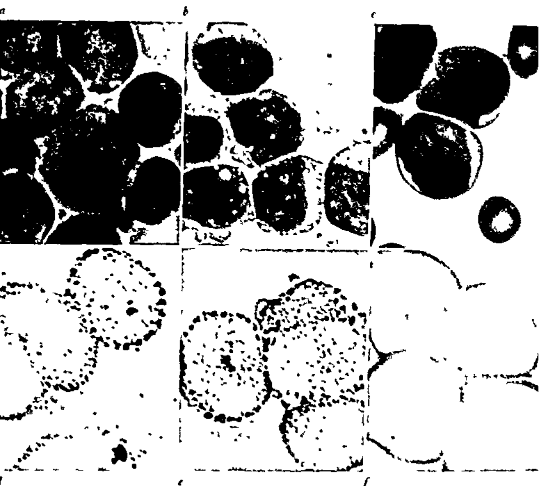


Fig. 1. Wright's stain and PAS reaction in the primitive cells of the acute leukemias. Wright's stain: *a* Lymphoblasts from ALL, *b* monoblasts from AML, *c* myeloblasts from AGL. PAS reaction: *d* PAS reaction in lymphoblasts from ALL exhibiting fine and coarse granules and clumps of reactive material, *e* PAS reaction in monoblasts from AML demonstrating fine and coarse granules, *f* PAS reaction in myeloblasts from AGL showing diffuse staining (× 1,500).

strong coloration (+−++), and myeloblasts of AGL weak to moderate activity (+−++) (table I). PAS reaction patterns in lymphoblasts of ALL show fine to coarse particulate granules with coarse clumps of positive material frequently seen, monoblasts of AML exhibit diffuse staining, fine and coarse granules, but no clumps of reactive material, myeloblasts of AGL are often nonreactive or show diffuse coloration with an occasional cell possessing particulate

glycogen granules. Most often, the reactive granules have a peripheral cytoplasmic distribution (fig. 1d–f).

Generally, the degree of phosphorylase activity and its intracellular localization in the primitive cells of the acute leukemias corresponds closely with the cytoplasmic distribution and the relative amount of preformed glycogen demonstrated by the PAS reaction (table I). On occasion, individual blast cells from the acute leukemias show a more intense phosphorylase reaction than would be expected from their PAS reactivity.

Sudan black B reaction in lymphoblasts of ALL exhibits weak to moderate staining (+ – +), monoblasts of AML moderate to intense reaction (+ + – + + + –), and myeloblasts of AGL moderate to strong coloration (+ [illegible] + [illegible] +) (table I). Reaction patterns in lymphoblasts of ALL appear as small sudanophilic granules localizing near the nuclear hof with many cells nonreactive, in monoblasts of AML as small and large sudanophilic granules depositing randomly in the cytoplasm with some cells exhibiting areas of intense activity, and in myeloblasts of AGL as small and moderately large sudanophilic granules localizing near the periphery of the cytoplasm (fig. 2a–c).

Acid phosphatase activity in lymphoblasts of ALL shows weak to moderate activity (+ – + +) and monoblasts of AML and myeloblasts of AGL demonstrate moderate to strong reactivity (+ + + + + + +) (table I). The reaction granules range in size from small to large granules with a cytoplasmic localization more intense near the nuclear hof (fig. 2d–f).

Primitive cells in leukemia, like the normal precursor cells, are negative for alkaline phosphatase activity. As alkaline phosphatase reaction is demonstrable in the mature neutrophils of the patients with acute leukemias, the neutrophils may be used in assessing levels of leukocyte alkaline phosphatase reactivity. The reactivity is visible as fine granules with the majority of neutrophils in ALL exhibiting strong to intense activity (+ + + – + + + +), in AML showing weak to strong reactions ([illegible] +), and in AGL demonstrating weak activity (+) with numerous cells nonreactive. Many of the *neutrophils* in AGL appear to have alterations in size and *number of specific* granules, but an occasional neutrophil will exhibit moderate activity (table I).

Nonspecific esterase reaction in lymphoblasts of ALL has weak to moderate reactivity (+ – + +) with fine to coarse granules localizing

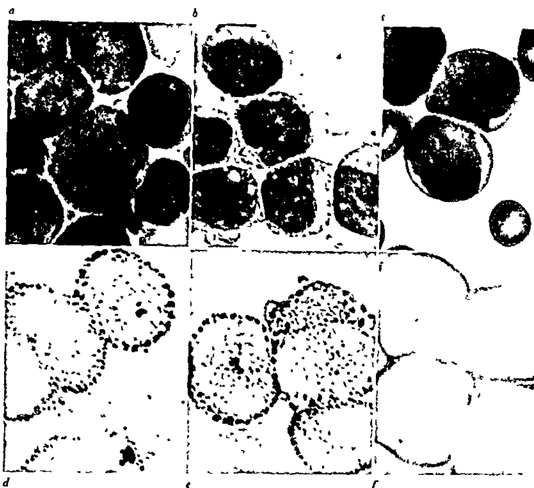


Fig 1 [illegible] stain [illegible] PAS re[illegible] granule[illegible] clumps of reactive material, *e* PAS reaction in monoblasts from AML demonstrating fine and coarse granules, *f* PAS reaction in myeloblasts from AGL showing diffuse staining (× 1,500)

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Nonspecific esterase reaction in lymphoblasts of ALL has weak to moderate reactivity ([illegible]) with fine to coarse granules localizing

Table I Summary of cytochemical reactivity and mean scores of primitive cells of patients with acute leukemias and blood and bone marrow cells of normal controls Reactivity rated as weak, moderate, strong, and intense

Cytochemical reaction	Cell type	Reactivity				Mean score
		wk	mod	str	int	
PAS	L-lymphoblast		++	+++	++++	134
	N lymphocyte	+	++			39
	L-monoblast	+	++	+++		88
	N-monocyte	+	++	+++		74
	L-myeloblast	+	++			53
	N-myeloblast	+				35
Phosphorylase	L-lymphoblast		++	+++	++++	120
	N lymphocyte	+	++			38
	L-monoblast	+	++	+++		84
	N-monocyte	+	++	+++		71
	L-myeloblast	+	++			46
	N myeloblast	+				33
Sudan black B	L-lymphoblast	+	++			56
	N-lymphocyte	+	++			54
	L-monoblast		++	+++	++++	142
	N-monocyte		++	+++		131
	L-myeloblast		++	+++		127
	N-myeloblast	+	++			96
Nonspecific esterase	L-lymphoblast	+	++			71
	N-lymphocyte	+	++			68
	L-monoblast		++	+++	++++	144
	N-monocyte		++	+++		140
	L-myeloblast	+	++			75
	N-myeloblast	+	++			73
Acid phosphatase	L-lymphoblast	+	++			90
	N-lymphocyte	+	++			94
	L-monoblast		++	+++		128
	N-monocyte		++	+++		132
	L-myeloblast		++	+++		120
	N-myeloblast	+	++			86
Alkaline phosphatase	L-neutrophil (ALL)	+	++	+++	++++	180
	L-neutrophil (AML)	+	++	+++		91
	L-neutrophil (AGL)	+	++			25
	N neutrophil	+	++	+++		82

L Leukemic, *N* normal

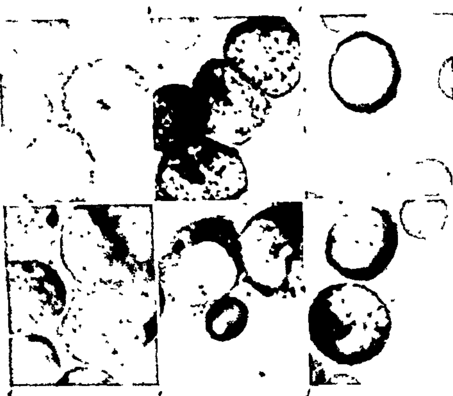


Fig. 2. Sudan black reaction and acid phosphatase activity in the primitive cells of the acute leukemias. Sudan black reaction: *a* Lymphoblasts from ALL showing a few reactive granules localizing primarily near the nuclear hof. *b* Monoblasts from AML demonstrating fine and coarse granules scattered throughout the cytoplasm. *c* Myeloblast from AGL showing fine to moderately coarse granules localizing near the periphery of the cytoplasm. Acid phosphatase: *d* Lymphoblasts from ALL demonstrating localization in small and large granules with most intense activity near the nuclear hof. *e* Monoblasts from AML exhibiting reactivity in small and large granules depositing more intensely near the nuclear hof. *f* Myeloblasts from AGL showing more intense activity in a perinuclear position. ×1,500.

primarily in the perinuclear cytoplasm. Monoblasts of AML show moderate to intense reaction (+++ to ++++) with heavier and coarser granules depositing randomly in the cytoplasm. Myeloblasts of AGL exhibit weak to moderate activity (+ to ++) with fine to moderately coarse granules scattered throughout the cytoplasm (table I), (fig. 3a–c).



Fig 3 Nonspecific esterase activity in the primitive cells of the acute leukemias *a* A lymphoblast from ALL showing fine and coarse granules localizing primarily near the nuclear hof, *b* monoblasts from AML exhibiting fine and coarse granules depositing randomly throughout the cytoplasm, *c* myeloblasts from AGL demonstrating fine and moderately coarse granules scattered throughout the cytoplasm (×1,500)

In general, the cytochemical reactivity and reaction patterns in normal blood and bone marrow cells are similar to those observed in the primitive cells of the acute leukemias except that the reactivity often appears less intense. However, the alkaline phosphatase reaction in normal mature neutrophils shows strong activity in a majority of the cells (table I). In normal lymphocytes, no clumps of PAS reactive material can be observed and no particulate PAS reactive granules appear in normal myeloblasts Sudan black B reaction in myeloblasts of AGL shows greater reactivity than that observed in normal myeloblasts

Discussion

Cytological and electron microscopic cytochemical studies reveal that acid phosphatase and nonspecific esterase activities localize in azurophilic and specific granules of leukocytes [3, 12], alkaline phosphatase activity in the specific granules of mature neutrophils [2, 3], and lipids in the mitochondria and cytoplasmic granules of leukocytes [2, 12] Comparison of the mean amounts of acid phosphatase, nonspecific esterase, and Sudan black B reactions of the primitive cells of ALL and AML to mature normal cells and myeloblasts of AGL to


Anti-A

normal myeloblasts suggests that a more advanced cytoplasmic differentiation of the primitive cells has occurred with numerous cytoplasmic granules and mitochondria appearing at an earlier time in maturation. The altered levels of alkaline phosphatase activity seen in the mature neutrophils of AGL may result from the qualitative and quantitative difference observed in the specific granules, while the higher levels appearing in the mature neutrophils of ALL and AML may denote their response to infections and stress situations [19].

In the present study, the significantly greater mean amounts of PAS reaction observed in the primitive cells of the acute leukemias as compared to normal controls is not in agreement with an earlier investigation [8] which reveals no significant differences in the glycogen content of these cells. Moreover, the results support other evidence [11] which shows that the PAS reactive material in myeloblasts of AGL is as intense as normally seen in promyelocytes and myelocytes. It has been suggested that the glycogen increase in leukemia may be compatible with an increase in the proliferative and metabolic activity of the lymphocytic system as seen in other nonleukemic diseases [15]. It is conceivable that in leukemia the presence or absence of specific enzymes and or organelles concerned with the enzymatic control of glycolysis may be responsible for the increased amounts of glycogen seen in the primitive cells. The present study and other investigations [11, 21] suggest that the increased levels of phosphorylase activity parallel closely the increased amounts of preformed glycogen. These observations tend to show that the increased levels of glycogen may result from increase in the levels of phosphorylase activity.

The primitive cells of the patients in the acute phase of chronic granulocytic leukemia do not show significantly different enzyme reaction patterns of the acid phosphatase, nonspecific esterase, or Sudan black B reactions as compared to the primitives cells of untreated patients with AGL.

Biochemical analysis of the leukocytes of the acute leukemias shows lower levels of alkaline and acid phosphatase activities as compared to higher levels found in normal controls [4, 18]. It has been stated that the lower levels of alkaline and acid phosphatase reactions observed is attributable to the low phosphatase activity of the primitive cells with increased activity appearing with maturation [4]. Cytochemically, the present study shows that small and significant differences in alkaline and acid phosphatase activities may be observed in the neutro-

phils and primitive cells, respectively, of the acute leukemias. The cytochemical results agree with biochemical observations which indicate that nonspecific esterase activity is similar in the primitive cells of the acute leukemias and normal blood and bone marrow cells [10, 17]. Sudan black B reaction for lipids tends to support the biochemical results of increased lipase activity in AGL and decreased lipase activity in ALL [13, 14].

PAS and Sudan black reactions and nonspecific esterase, acid phosphatase, and alkaline phosphatase activities may be valuable in the differentiation and classification of the primitive cells of the acute leukemias. Comparisons of the mean amounts of these reactions suggest their usefulness in the diagnoses of the acute leukemias. Moreover, our cytochemical evidence indicates that the primitive cells of the acute leukemias, which exhibit morphological and cytochemical variations, can be distinguished as specific types and are not modifications of a single precursor cell. The cytochemical reactions show sufficient variations in their mean scores and reaction patterns to suggest the existence of several varieties of stem cells.

Acknowledgements Dr CHRIS J D ZARAFONETIS of Simpson Memorial Institute, University of Michigan, and Dr G ADOLPH ACKERMAN of the Department of Anatomy, Ohio State University, gave helpful suggestions and advice, Dr INTA J ERTEL and Miss AGNES I. FRITZELL provided the blood and bone marrow specimens used in the study.

Summary

Blood and bone marrow films of 71 patients with acute leukemias and 21 controls were evaluated morphologically and cytochemically A semiquantitative scoring method was used for estimating the intensity of the cytochemical enzyme reactions Variations in the level of scores and reaction patterns of the acid phosphatase, nonspecific esterase, and alkaline phosphatase activities and the Sudan black and PAS reactions may provide additional criteria for the separation and classification of the acute leukemias Altered enzyme patterns exhibited by the primitive cells of the acute leukemias correlate closely with the degree of cytoplasmic and granular development and/or maturation Morphological and cytochemical variations indicate that the primitive cells of the acute leukemias represent specific varieties of stem cells

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Gastric Mucosa in Iron Deficiency Anaemia

Results of Follow-Up Examinations

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We have previously [1] described the findings in gastric biopsies of 100 patients with iron deficiency anaemia. In 47 of the patients a second gastric biopsy was performed after approximately 6 months' iron treatment. Slight changes (improvement or worsening) were seen in some patients, but in general the state of the gastric mucosa had remained unchanged. It seemed possible, that a more prolonged follow-up would throw some light upon the complex relations between iron deficiency anaemia and gastritis.

The present study concerns the gastric biopsy findings in 48 of our original 100 patients 7 years after the first examination.

Series and Methods

The series consists of 48 patients, 45 females and 3 males, who attented the follow-up examination to which all the 100 patients of our original series were invited.

The mean age of the series at the first examination (94 females and 6 males) was 35.3 years and that of the patients of the present series 36.5 years. Nine out of 25 patients with a normal gastric mucosa at the first examination, 25 out of 39 patients with superficial gastritis and 14 out of 36 with atrophic gastritis were re-examined.

There were no significant differences between the original and the present series in the number of patients with diseases of obvious significance for the development of iron deficiency. In 18 patients of the present series the case histories revealed factors clearly influencing the iron balance: intestinal malabsorption in 2, ulcerative colitis in 2, and profuse menstrual, haemorrhoidal or nasal bleedings in 14 patients.

The haematological examination consisted of a complete peripheral blood picture, bone marrow examination including sideroblast staining, serum iron and total iron binding capacity determinations, and serum vitamin B_{12} determination.

The acidity of the gastric contents was determined by maximal Histalog® test (2 hours' continuous suction after 2 mg/kg betazole).

Gastric biopsies were performed with the blind suction biopsy tube and in all 148 specimens were obtained. The specimens seemed to be representative of the state of the whole

gastric body mucosa in that there was a close correlation between the state of body glands and the maximal acid output (mean maximal acid output in patients with normal gastric mucosa was 7.6 mEq/h, in patients with superficial gastritis 9.6 mEq/h, in patients with partial loss of normal glands 0.7 mEq/h, and in patients with total loss of normal body glands 0 mEq/h.

Results

In the course of the follow-up 30 of the 43 patients had had one or more anaemic episodes and had received iron medication more or less regularly. Normal haemoglobin values had been consistently found in only 13 patients, and of the remaining 5 patients no examinations had been performed. The occurrence of anaemia did not correlate with the bioptical findings at the first examination. Thus anaemic episodes had been present in 6 of 14 patients with atrophic gastritis and in 7 of 9 patients with normal gastric mucosa at the first examination.

At the present examination only 19 patients were non-sideropenic, while 12 had distinct iron deficiency anaemia and 17 sideropenia with haemoglobin levels over 12 g/100 ml. One of the non-sideropenic patients fulfilled the criteria of Addisonian pernicious anaemia. There was no definite correlation between various haematological parameters and the bioptical state of the gastric mucosa at the present examination.

The changes occurring in the state of the gastric mucosa during the follow-up period are shown in table I. It appears that the state remained unchanged in 33, got worse in 12 and improved in 3 patients. In table II the changes in the state of the mucosa during the follow-up period are correlated with the haematological findings during the follow-up and at the present examination. Some correlations are seen: 2 of the 3 patients histologically improved had not been anaemic during the follow-up and none of them was anaemic at the present examination although two were sideropenic, while 9 of the 12 patients with progression of mucosal changes had had anaemia during the follow-up (the remaining 3 had not been examined) and 5 of them had iron deficiency anaemia at the present examination. Moreover it should be noted that gastritis was found in 81% of the patients re-examined, while the prevalence of gastritis in randomly selected subjects of a Finnish rural population of almost corresponding mean age was 52% [2]. On the other hand, the occurrence of atrophic gastritis showed a rather clear correlation with the age of the patients: at the present examination the median age of patients with normal gastric mucosa was 30 years (22–59 years), that of patients with superficial gastritis 39 years

Table I Gastric biopsy findings in 48 patients studied in 1962 and 1969

;astric biopsy .nding in 1962	Gastric biopsy finding in 1969				
	Normal	Superficial gastritis	Slight atrophic gastritis	Moderate atrophic gastritis	S. a: g:
ıormal	6	3	–	–	–
uperficial gastritis[1]	1	21	–	2	1
light atrophic gastritis[2]	2	–	1	4	–
Moderate atrophic gastritis[3]	–	–	–	3	2
evere atrophic gastritis[4]	–	–	–	–	2

[1] Increased round cell infiltration with normal body glands
[2] Slight loss of normal body glands.
[3] Moderate loss of normal body glands
[4] *Complete loss of normal body glands or only remnants of them present*

'able II Median age of the patients, their distribution by the occurrence of anaemia during the foll period and by the haematological findings in 1969 according to the change in gastric biopsy finc

;astric ıopsy nding	Median age in 1962 years	Haemoglobin level during follow up			Haematological finding 1969		
		Anaemic	Not studied	Normal	Iron deficiency anaemia[1]	Sideropenic[2]	: s r
mproved	20 (20–47)	1	–	2	–	2	
Inchanged	34 (15–70)	20	2	11	7	12	1
Vorsened	41 (24–54)	9	3	–	5	3	

[illegible] capacity
[illegible] len
ount below 10%

(25–57 years), and that of patients with atrophic gastritis 56 years (31–77 years).

In a 47-year-old female patient the disappearance of slight atrophic gastritis was accompanied by reappearance of acid secretion. In a 34-year-old female patient the progression of moderate atrophic [illegible] the develop-

Serum vitamin B_{12} levels were normal in all but 2 patients: the one with pernicious anaemia and another with intestinal malabsorption.

Discussion

The most striking observation was the relapsing tendency of sideropenia, although measures to prevent the recurrence of iron deficiency anaemia were stressed to each patient at the first examination. Obviously the original, known and unknown, causes of iron deficiency had persisted and the treatment given had not been sufficient to overcome these. There was no evidence to suggest that atrophic gastritis had predisposed to relapses of iron deficiency. On the other hand, normalization of the histological features occurred mainly in those who had not been anaemic during the follow-up, whereas most of the patients with worsened mucosal findings had been anaemic and showed signs of iron deficiency. In one patient the normalization of gastric mucosa was accompanied by the return of acid secretion. Hence, the evidence available does not favour the primary significance of gastritis in the development of iron deficiency. In contrast they suggest that iron deficiency predisposes to alterations of the gastric mucosa. The present follow-up series is, however, too small and the bioptical method not reliable enough to permit any definite conclusions. The judging of the results is, in addition, complicated by the age-dependent progression of gastritis [2].

Summary

The fate of the gastric body mucosa was followed-up for about 7 years in 43 out of 100 bioptically examined patients with iron deficiency anaemia. During the follow-up period 30 of the 43 patients had one or more anaemic episodes, and at reexaminations 12 were found to have iron deficiency anaemia and an additional 17 sideropenia without anaemia. The occurrence of anaemia did not correlate with bioptical findings at the first examinations. At the reexamination the state of the gastric mucosa was found to have improved in 5, got worse in 12 and remained unchanged in 33. Anaemic episodes were more frequent in those who showed a progression of gastritis than in those improved.

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Zur Proliferationskinetik der Lebererythropoese heranwachsender Ratten[1]

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Die neonatale Hämopoese der Ratte wird zur Zeit der Geburt beinahe ausnahmslos von extramedullären Ursprungsorten gespeist. Dabei ist eine der Hauptproduktionsstätten die Leber. Die Untersuchung der Entwicklungskinetik der Lebererythropoese erschien lohnend, da entsprechende Untersuchungen zur Proliferationskinetik der neonatalen Knochenmarkerythropoese in unserer Gruppe erarbeitet wurden [11, 12]. Ein Vergleich der Lebererythropoese in den ersten Wochen nach Geburt mit der des Knochenmarkes liess Hinweise uber unterschiedliche Proliferationsmodi erwarten. Ein derart unterschiedlicher Zellumsatz kann vermutet werden, da es sich im einen Fall um ein untergehendes, im anderen Fall um ein im Aufbau befindliches Zellsystem handelt.

Zur Untersuchung dieser Fragen wurde eine zytokinetische Methode angewandt, die es ermoglicht, mittels radioaktiven Thymidins (^{3}H-TdR) *alle* Zellkerne zu markieren, auch diejenigen, die eine nur sehr langsame Proliferationsaktivität aufweisen und daher zytokinetisch als «Ruhezellen» bezeichnet werden können [8, 9].

Material und Methoden

Versuchstiere Als Versuchstiere dienten 4 Monate alte Wistarratten mit einem durchschnittlichen Körpergewicht von 180 g Der Ovulationszyklus wurde durch Vaginalab-

[1] Die Untersuchungen wurden durch die Euratom-Verträge «Strahlenhämatologie» Nr 031-64-1-BIAD und 072-68-1-BIOD unterstützt

[2] Wesentliche Teile dieser Arbeit entstammen der Dissertation von E. SPARRER, Universität Ulm

strahe bestimmt [18]. Die Kopulation wurde über Nacht herbeigeführt. Als Beginn der Gravidität galt 0.00 Uhr dieses Tages, wenn im Vaginalabstrich Spermien nachweisbar waren.

*³H-TdR-Applikation*¹. Den schwangeren Ratten wurde vom 9. Tag der Gravidität an (entsprechend dem Beginn der Organogenese [13]) bis zum Geburtstermin 1,5 µCi/g/Tag ³H-Thymidin in physiologischer Kochsalzlösung verabreicht. Die spezifische Aktivität des ³H-TdR betrug 2,0 Ci/mM. Die Applikation des ³H-TdR erfolgte bei der Hälfte der Tiere alle 6 Stunden i.p. Die andere Hälfte erhielt ³H-TdR kontinuierlich über die Schwanzvene mittels eines Polyäthylen-Katheters (modifizierte Methode nach Lewis et al. [15]). Da hinsichtlich des Markierungserfolges bei beiden Methoden kein Unterschied festgestellt wurde [9], wurden die Tiere im folgenden jeweils zusammengefaßt.

Tötung der Tiere. Ausgewertet wurden insgesamt 51 junge Ratten. Die Tötung der Tiere erfolgte 1, 2, 6, 12, 24, 30, 50 Stunden, 3, 4, 6, 10 Tage und 2, 4, 6, 8, 10, 21 Wochen nach der Geburt.

Aufarbeitung des Materials. Von der Leber wurden jeweils histologische und zytologische Präparate angefertigt. Die Autoradiographie der Tupfpräparate erfolgte mit der Stripping-film (Kodak AR 10)-Methode. Die histologischen Präparate wurden mit einer flüssigen Fotoemulsion (Ilford L4) beschichtet. Die Expositionszeit für die zytologischen Präparate betrug 9, für die histologischen 45 Tage. Nach üblicher Entwicklung und Fixierung wurden die Tupfpräparate mit Giemsa bei einem pH von 5,75, die histologischen Präparate mit Hämatoxylin-Eosin gefärbt.

In den Tupfpräparaten kamen von jedem Tier zu jedem Zeitpunkt für die zytologische Identifizierung und für die Untersuchung der Markierungsverhältnisse mindestens 200 erythropoetische Zellen zur Auswertung. Die zytologische Klassifizierung der Erythropoese erfolgte in Anlehnung an das Schema von Weiss [21]. In den histologischen Präparaten wurde zu verschiedenen Zeitpunkten der Entwicklung der Tiere nach Geburt das Verhältnis der einzelnen Erythroblasten verschiedener Reifungsstufen zueinander ermittelt. Dafür kamen pro Zeitpunkt und Tier mindestens 200 Zellen zur Auswertung, die in 5 [illegible] (1430facher Vergrößerung) ausgezählt wurden.

Ergebnisse

In den zytologischen und histologischen Präparaten der Leber waren *alle* Zellkerne markiert, wenn die Tiere kurz nach der letzten ³H-TdR-Applikation getötet wurden. Bei allen Neugeborenen war diese 100%ige Markierung der Zellen vorhanden, unabhängig von der angewandten Methode der ³H-TdR-Applikation. Die Markierungsintensität in den zytologischen Tupfpräparaten schwankte zwischen 20–70 Körnchen über den verschiedenen Zellen der Erythropoese. Es fand sich keine Zelle mit weniger als 5 Körnchen.

Entwicklung der Leberhämopoese nach Geburt. Die Entwicklung der neonatalen Rattenleber ist durch allmähliches, auch makroskopisch sichtbares Zurückgehen der hämopoetischen Funktion dieses Organs zugunsten der Ausbildung des eigentlichen Leberparenchyms gekennzeichnet. Interessanterweise sind die Lebergewichte neugeborener

¹ ³H-TdR vom Radiochemical Center, Amersham, England.

Ratten in den ersten 6 Tagen nach Geburt relativ konstant zwischen 0,2 und 0,3 g, d.h., dass in den ersten Tagen keine Gewichtszunahme der Leber gegenüber dem Geburtsgewicht feststellbar ist. Erst ab dem 6. Lebenstag kommt es dann zu einem linearen Anstieg des Lebergewichtes, der bis 6 Wochen nach Geburt verfolgt wurde. Dabei ist in den ersten Lebenstagen eine deutliche mitotische Aktivität des Leberparenchyms erkennbar. Die neonatale Lebergewichtsentwicklung resultiert somit aus dem Verschwinden der Hämopoese und der Zunahme der Leberzellen.

Die histologischen Präparate lassen folgende Entwicklung erkennen: Zur Zeit der Geburt ist die typische Leberstruktur mit Läppchenaufbau noch nicht ausgebildet, vielmehr dominieren herdförmig oder dissiminiert im Leberparenchym gelegene erythropoetische Zellen. Daneben finden sich myelopoetische Zellen, meist um Lebergefässe gelagert. Vereinzelt werden auch Megakaryozyten inmitten des Leberparenchyms gesehen. Innerhalb der ersten Lebenswoche kommt es zu einem allmählichen Rückgang der Leberhämopoese. Dabei liegen jetzt die erythropoetischen Zellen nur noch in kleinen Inseln vor. In dieser Zeit verschwindet die Myelopoese und Megakaryozytopoese vollständig. Zwei Wochen nach Geburt ist die typische Leberläppchenstruktur ausgebildet. Kleine erythropoetische Herde sind ohne besondere topographische Beziehung im Parenchym verstreut. Drei Wochen nach Geburt ist auch in der Leber keine Erythropoese mehr sichtbar.

In Abbildung 1 sind die zahlenmässigen Veränderungen der Erythropoese im Verhältnis zu den Leberparenchemzellen in Abhängigkeit von der Zeit nach Geburt dargestellt. Daraus ergibt sich, dass unmittelbar nach Geburt etwa 50% aller Zellen der Leber zur Erythropoese gehören und dass dieser Anteil innerhalb von 10 Tagen auf weniger als 10% abfällt.

Es war nun von besonderem Interesse zu untersuchen, ob sich das Verhältnis der erythropoetischen Zellen zueinander in den ersten Tagen nach Geburt ändert. Ein plotzliches Aufhören der Proliferation und Differenzierung von Stammzellen in erythropoetische Vorstufen müsste ein deutliches Verschwinden der unreifen Erythroblasten lange vor dem Zurückgehen der reiferen Zellformen zur Folge haben. In Abbildung 2 sind die Prozentzahlen der einzelnen Reifungsstufen der Erythropoese im Verhältnis zur Gesamterythropoese zu den verschiedenen Zeiten nach Geburt eingetragen, die in Lebertupfpräpara-

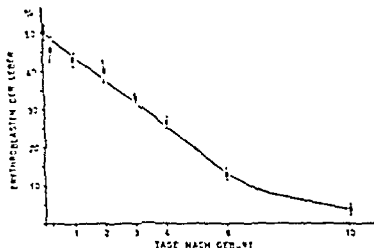


Abb. 1 Änderung der relativen Erythroblastenanzahl in der Leber als Funktion der Zeit. Jeder Punkt der Kurve repräsentiert mindestens 5 Tiere. Der Standardfehler ist angegeben

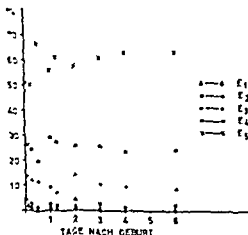


Abb. 2 Prozentualer Anteil der einzelnen erythropoetischen Reifungsstufen in der Leber zu verschiedenen Zeitpunkten nach Geburt.

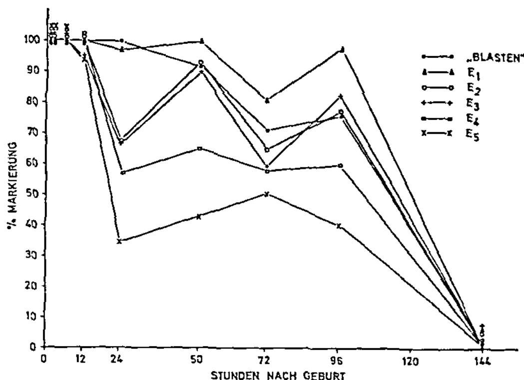


Abb 3 Markierungsverhalten der Lebererythropoese als Funktion der Zeit

ten ermittelt wurden. Dabei zeigt sich, dass sich bis zum 6 Tag nach Geburt das Verhältnis der einzelnen Zellklassen zueinander nicht verändert. Fasst man nun sämtliche Zellen (insgesamt wurden 31500 Zellen ausdifferenziert) der verschiedenen Reifungsstufen zu allen ausgewerteten Zeitpunkten nach Geburt zusammen und setzt sie miteinander in Beziehung, so ergibt sich, dass von 500 pro Präparat ausgezählten Zellen im Mittel 5 E_1-Zellen, 15 E_2-Zellen, 60 E_3-Zellen, 132 E_4-Zellen und 288 E_5-Zellen ausgezählt wurden entsprechend 1% E_1-, 3% E_2-, 12% E_3-, 26% E_4- und 58% E_5-Zellen Aus dieser Verteilung der einzelnen erythropoetischen Vorstufen in der neonatalen Leber ergibt sich fast eine Verdoppelung der Zellen von einer Reifungsstufe zur nächsten.

Proliferationsverhalten erythropoetischer Zellen der Leber bei vollständig markierten neugeborenen Ratten Das Markierungsverhalten der Lebererythropoese wurde in Tupfpräparaten, die autoradiographisch behandelt waren, untersucht Neben den 5 Klassen kernhaltiger erythro-

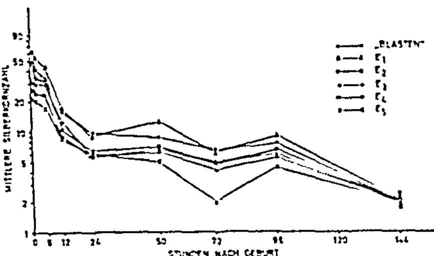


Abb. 4. Markierungsintensität der Lebererythropoese als Funktion der Zeit

poetischer Vorstufen wurde eine Zellgruppe «undifferenzierter Blasten» unterschieden. Diese werden von einigen Autoren als mögliche Vorläufer der Erythropoese angesehen [16, 17].

Der Abfall des Markierungsindex über diesen Zellpopulationen in Abhängigkeit von der Zeit ist in Abbildung 3 aufgetragen: Die Blasten bleiben bis 24 h p.p. 100% markiert. Anschliessend kommt es zu einem stetigen Abfall des Markierungsindex, bis nach 6 Tagen praktisch keine Blasten mehr markiert sind. Ein ähnlicher Verlauf findet sich auch bei den Markierungsindices der übrigen erythropoetischen Vor- [illegible] Zellpopulationen praktisch nicht mehr als markiert zu erkennen. In Abbildung 4 sind die Veränderungen der Markierungsintensität der hämopoetischen Blasten und verschiedenen Vorstufen der Erythropoese dargestellt. Daraus geht hervor, dass die mittlere Silberkornzahl der noch undifferenzierten Blasten in den ersten 12 Stunden p.p. von etwa 50 auf etwa 15 Körnchen pro Zelle abfällt. Danach fallen die Werte langsamer ab bis auf im Mittel 2 Silberkörner pro Blastkern 6 Tage p.p. Die erythropoetischen Zellvorstufen E_1–E_4 zeigen einen

ähnlichen Verlauf: Auch bei allen diesen Zellkategorien kommt es innerhalb der ersten 12 Stunden p.p. zu einem rapiden Abfall der Markierungsintensität gefolgt von einem langsameren Abfall, bis 6 Tage p.p. nur noch wenige Körnchen über den Zellen erkennbar sind. Eine zeichnerische Subtraktion der beiden Komponenten der Kurven ergab folgende Halbwertzeiten für den Abfall der Markierung im ersten Teil: Blasten ca. 3 h, E_1 ca. 3,5 h, E_2 ca. 6 h, E_3 ca. 5 h, E_4 ca. 4 h und E_5 ca. 7 h.

Diskussion

Die vorgelegten Befunde demonstrieren die Möglichkeit, durch eine 100%ige Markierung der Zellkerne neugeborener Ratten, die Umsatzkinetik schnell proliferierender Zellen wie etwa die der Lebererythropoese zu untersuchen. Da zu Beginn der Untersuchung alle Zellen markiert waren, kann der Abfall der Thymidinmarkierung über den Zellen als Mass für den Zellumsatz gelten, ohne dass zunächst der Einstrom eines vorgeschaltenen nichtmarkierten Zellsystems berücksichtigt werden musste.

Voraussetzung zur Anwendung der 100%igen Markierung ist der Nachweis, dass die angewandten ^{3}H-TdR-Dosierungen keine offensichtliche oder latente Schädigung der zu untersuchenden Zellpopulation herbeiführten. Die bisher gesammelten Befunde zu dieser Frage sind an anderer Stelle ausführlich dargestellt worden [8–10]. Fur die hier angewandten Dosen kann davon ausgegangen werden, dass keine Einflüsse durch die Radiotoxizität des Tritium-Thymidins berücksichtigt werden müssen.

Weiterhin musste geprüft werden, inwieweit eine Reutilisation von ^{3}H-TdR markierten DNS-Bausteinen zustande kommt [2, 7, 14, 19]. Ein solcher Einfluss ist gerade durch den allmählichen Untergang rasch proliferierender hämopoetischer Zellsysteme in der Leber gut denkbar. Die Untersuchung dieser Frage [ausführliche Darstellung s. 20] ergab eine deutliche Beeinflussung des Abfalls der Markierungsintensität aller erythropoetischen Zellen und der undifferenzierten Blasten. Eine weitgehende Ausschaltung der Reutilisation durch wiederholte Applikation von inaktivem Thymidin änderte jedoch nichts am prinzipiellen Kurvenverlauf für den Markierungsindex und die Markierungsintensität. Diese Beobachtung steht in guter Übereinstimmung mit den Befunden, die wir für schnell proliferieren-

de Zellsysteme des Knochenmarkes erheben konnten [9]. Bei beiden Zellsystemen spielen Reutilisationsprozesse von ^{3}H markierter DNS oder deren Bausteine eine etwa gleich starke Rolle.

Der rasche Umsatz der Erythropoese des Knochenmarkes ist wohl bekannt [3–6]. Die vorliegenden Untersuchungen sollten nun zum Verständnis der Proliferationskinetik eines schnell proliferierenden Systems dienen zu einem Zeitpunkt, zu dem dieses abgebaut wird. Es konnte im Vorhergehenden nachgewiesen werden, dass während der neonatalen Entwicklung der Rattenleber die verschiedenen Reifestufen der Erythropoese im gleichen Verhältnis zueinander verschwinden. Dieser Befund spricht dafür, dass die Umsatzgeschwindigkeit der Erythropoese der Leber in allen kernhaltigen Reifungsklassen *allmählich* abnimmt. Wenn nämlich ein plötzliches Aufhören der Differenzierung und Proliferation einer Vorstufe, z. B. der Blasten, einträte, müsste ein Verschwinden der Zellformen im Sinne einer auslaufenden »Pipeline« erwartet werden, d. h., es müsste zunächst die L_1-Population, dann die L_2-Population usw. verschwinden, ein Vorgang, der nicht beobachtet werden konnte. Da jedoch andererseits kein vermehrtes Absterben erythropoetischer Zellen in der Leber auftritt, muss man annehmen, dass bei verlangsamter Produktion reife Normoblasten mit etwa gleicher Geschwindigkeit ins Blut abströmen wie zu früheren Entwicklungsstadien, so dass sich das Kompartiment der Erythropoese langsam entleert. Dass tatsächlich Normoblasten im peripheren Blut existieren, solange bei der Ratte extramedulläre Blutbildung vorhanden ist, konnte in anderen Untersuchungen nachgewiesen werden [1]. Die Beobachtung des Markierungsverhaltens während der Neonatalperiode erbringt ebenfalls die Bestätigung, dass es zu einer verlangsamten Proliferationsaktivität der ganzen Population kommt, bis diese endlich aus der Leber verschwindet. So konnte gezeigt werden, dass erythropoetischen Populationen einen Abfall der Markierungsintensität in den ersten 24 Stunden nach Geburt von mindestens 50% aufweisen, anschliessend aber ein wesentlich geringerer Abfall der Markierungsintensität bis zum 6. Lebenstag über diesen Zellen auftritt. Dieser prinzipielle Kurvenverlauf ist auch unter Berücksichtigung der ^{3}H TdR-Reutilisation nachweisbar. Ein Vergleich der Markierungswerte der Lebererythropoese mit denen des Knochenmarkes der gleichen Tiere [11, 12] zeigt, dass 24 h nach Geburt im Knochenmark im Gegensatz zur Lebererythropoese noch alle erythropoetischen Zellen 100% markiert sind. Der Abfall der

Markierungsintensität über E_1-Zellen des Knochenmarkes tritt mit einer Halbwertzeit von etwa 12 h erst viel später als in der Leber auf, nämlich 24 h nach Geburt. Die Lebererythropoese verhält sich also zytokinetisch anders als die sich entwickelnde Knochenmarkerythropoese wie zu erwarten war [Einzelheiten s 12]. Für die Zeit kurz nach Geburt kann aus der Betrachtung der Halbwertszeiten für den Markierungsabfall auf einen sehr schnellen Zellumsatz geschlossen werden, während 12 h p p eine starke Verlangsamung beobachtet wurde. Dieser Befund steht in guter Übereinstimmung mit den Ergebnissen von LUCARELLI *et al.* [18], der kurze Generationszeiten der pranatalen Lebererythropoese in der Grossenordnung von 4–6 h gefunden hat

Zusammenfassung

Durch kontinuierliche ^{3}H Thymidin Gabe während der Schwangerschaft wurde eine Markierung aller Zellkerne der Leber neugeborener Ratten ermöglicht Die Tötung der neugeborenen Ratten zu verschiedenen Zeiten nach Geburt erlaubte die zytokinetische Untersuchung der Lebererythropoese während der Neonatalperiode Es konnte gezeigt werden, dass die Lebererythropoese einer anderen Kinetik folgt als die Erythropoese des sich entwickelnden Knochenmarkes der gleichen Tiere In den ersten 12 Stunden p p weist die Lebererythropoese einen sehr raschen Zellumsatz auf, der sich anschliessend parallel zum Verschwinden der Zellpopulation, immer mehr verlangsamt

Summary

By repeated or continuous application of ^{3}H thymidine to rats during pregnancy, labelling of all cells of newborn liver was achieved The newborn rats were sacrificed at different times after birth and a study made of the subsequent development of erythropoietic cells The kinetics of erythropoiesis in the liver were found to differ from the kinetics of erythropoiesis in the developing bone marrow of the same animals. The very rapid cell turnover of erythropoietic liver cells during the first 12 h after birth is followed by a continous decrease of proliferative activity subsequent to the disappearance of erythropoiesis in the liver

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The Osmotic Fragility and Density Distribution of Erythrocytes in the Newborn[1]

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Various opinions have been expressed regarding the differences in osmotic fragility between normal newborn red blood cells as compared to the cells of normal adults. Goldbloom and Gotlieb [8] reported increased osmotic fragility of newborn red blood cells. Whitby and Hynes [13], cited by Wintrobe [14], claimed that the red blood corpuscles of newborn children are somewhat more fragile than those of adults, but did not regard the difference as *significant*. Waugh *et al.* [12] reviewed 15 papers on the subject and concluded that fetal cells are slightly more resistant. Their own experiments indicated that the main feature of fetal cells with regard to their osmotic fragility is an increased range and a slight tendency to increased resistance. Findlay [6], testing the salt concentration at which 50% hemolysis occurred, found that blood taken from the umbilical cord was *more resistant*. However, the difference was not apparent when the blood of the newborn was compared with that of the adult. Sjölin [10] reported similar results, emphasizing the presence of a population of *more resistant cells* in cord blood. This population of resistant cells gradually decreases until complete disappearance at the onset of puberty. In cattle, the [illegible] approximately one life span of [illegible] differences in methods employed and especially to the limited number of points which were used to construct the osmotic fragility curve.

[1] This work was submitted as a partial fulfillment of MD thesis in the Hebrew University Hadassah Medical School, Jerusalem.

In the present work, the osmotic fragility of blood from the umbilical cord is compared to that of blood from adults, using a method whereby the osmotic fragility curve is recorded, in great detail, on the basis of continuously decreasing salt concentrations [4]. Additional information was obtained by establishing the density distribution of the red blood cells (DDC) by differential flotation, using mixtures of phthalate esters of predetermined specific gravity as described by Danon and Marikovsky [5].

Materials and Methods

Blood was withdrawn from the umbilical cord of 100 newborn babies by opening the surgical clamp. After discarding the first few drops, the blood samples were collected into a test tube previously wetted with heparin. Newborns that subsequently developed hemolytic diseases are not included in these 100 samples.

Normal adult blood obtained from 50 laboratory and hospital staff members by venepuncture was transferred into heparinized test tubes after removal of the needle. The fragility curves were obtained by using a fragiligraph Model D-2 (Elscint Ltd., Haifa, Israel). A battery of 20 phthalate esters was used for DDC determinations (Cravit Co., Miles-Yeda Ltd., POB 1122, Rehovot, Israel).

All blood samples were tested within two hours after withdrawal. Cord blood was generally tested immediately.

Results and Discussion

It can be seen from figure 1 that fetal blood is generally more resistant to osmotic pressure than the blood of adults. The narrow overlapping region indicates that up to 90% hemolysis may occur in the different types of cells at the same salt concentration.

From the DDC (fig. 2) it is apparent that the umbilical cord blood has a considerable proportion of cells of lower specific gravity, indicating mainly higher hydration. The overlap is more marked than that observed in the fragiligrams. It should be noted that cells of a high specific gravity, similar to the densest cells of adult blood, may be found in cord blood, reaching sometimes up to about 10% of the cells. These may be cells of adult type as found to exist in cattle even before the actual birth of the calf [7]. They may also be senescent fetal type cells.

Blood from umbilical cord containing two distinct populations of cells as depicted in figures 3 and 4 may sometimes occur.

The increase in osmotic resistance generally observed in red cells from cord blood in this study is in contradiction with the increased

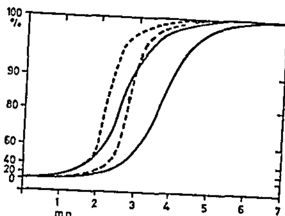


Fig 1 Range of normal adult (- - -) and newborn (——) fragiligrams Abscissa = time in minutes Ordinate = percent
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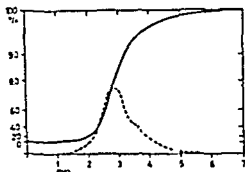


Fig 3. A fragilgram obtained from umbilical cord blood showing bimodality which is indicative of two cell populations, apparent in the derivative curve (- - -) as a shoulder on the right. It is not recognizable in the cumulative curve (——)

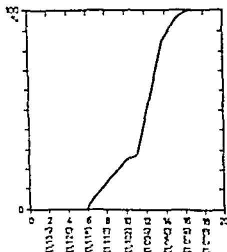


Fig 4. DDC obtained from umbilical cord blood of the same blood from which the fragilograms of figure 3 were obtained. Note the high proportion of cells with low specific gravity and the apparent bimodality

fragility reported by several authors [8, 13]. The data obtained by the 50% hemolysis test are of limited value as can be seen from the considerable amount of overlap in the osmotic resistance values for cord and adult blood at this level of hemolysis (fig 1). SJÖLIN [10]

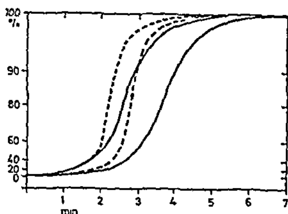


Fig 1. Range of normal adult (---) and newborn (——) fragiligrams Abscissa = time in minutes Ordinate = percent hemolysis The baseline was adjusted to hematocrite 50% using the nomograms supplied with the fragiligraph Note the narrow overlapping area between the two ranges and the relatively wide range of osmotic fragility of newborn blood

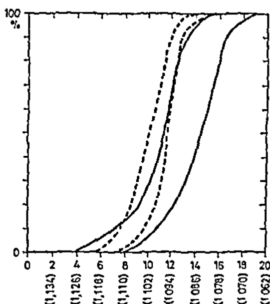


Fig 2 Range of normal adult (---) and newborn (——) DDC. Abscissa = specific gravity of separating fluid Ordinate = percent cells below separating fluid Note the relatively wide range of normal DDC of fetal blood and the existence of a small proportion of fetal red cells having a specific gravity similar or higher than that of the oldest or densest adult red blood cells.

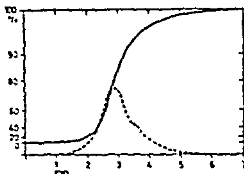


Fig. 3. A fragiligram obtained from umbilical cord blood showing bimodality which is indicative of two cell populations, apparent in the derivative curve (- - -) as a shoulder on the right. It is not recognizable in the cumulative curve (——)

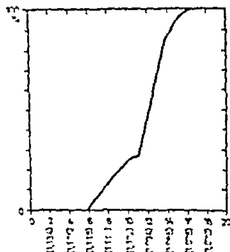


Fig. 4. DDC obtained from umbilical cord blood of the same blood from which the fragiligrams of figure 3 were obtained. Note the high proportion of cells with low specific gravity and the apparent bimodality

fragility reported by several authors [8, 13]. The data obtained by the 50% hemolysis test are of limited value as can be seen from the considerable amount of overlap in the osmotic resistance values for cord and adult blood at this level of hemolysis (fig. 1). Sjölin [10]

and CRAWFORD *et al* [3] have pointed out the presence of a small proportion of osmotically resistant cells in cord blood. They attributed this increased resistance to the relatively high proportion of reticulocytes in cord blood SMITH [11]noted that the proportion of cells with increased resistance is higher than 10%. However, our data indicate that the fetal cells hemolyse later, i e. at lower salt concentrations, than any reticulocytes of normal adults Also in the DDC, some cells show a specific gravity lower than any normal reticulocytes of adults These characteristics of cord blood cells seem to indicate a structural difference in addition to the higher concentration of fetal hemoglobin, as pointed out by ZIPURSKY *et al* [15]. Those differences are even more sharply delineated in cattle blood [3, 9]

It has been shown that, in the adult life of some mammals, a population of cells larger and more hydrated than the normal reticulocytes may sometimes occur [1] The characteristics of these cells correspond to the skip of one cell division, such cells would reach the circulation having larger diameters, and a higher surface to volume ratio and a higher proportion of water [1, 2] These features may contribute, at least partially, to the higher osmotic resistance and the lower specific gravity of these cells. Similar properties may cause the higher resistance of cells from cord blood In view of these results, it seems not unreasonable to assume that during life in utero, when the rate of blood formation is confronted with the needs of the body of the foetus and its high rate of growth, a high rate erythropoiesis and frequently 'skipped generation' may predominate [1]

Summary

The osmotic fragility of red cells from the newborn was compared with that of adult erythrocytes using a fragiligraph Model D-2 Density distribution of cells (DDC) of the two [illegible] of predetermined specific [illegible] f osmotic fragility [illegible] ing area with the [illegible] the blood of the new born babies is as a [illegible]

is of higher density than the densest cells of adults

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The Osmotic Fragility of Red Cells in Newborns and Infants

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Erythropoiesis undergoes a series of striking changes during development in mammals [9, 11], and human fetal cells differ from adult cells in numerous respects [reviewed by C. A. Smith, 17]. Analysis of osmotic fragility yields important information as to the homogeneity, the shape and the surface to volume ratio of a population of red cells. Earlier work has indicated that on the average newborn erythrocytes tend to be osmotically more resistant than in the adult but that abnormally fragile cells are also present [19]. Danon *et al.* [3] have recently confirmed these data by use of an automatic (fragiligram) technique [2]. In this paper, we show how the wider range of osmotic fragility is a unique characteristic of normal newborn cells which can be precisely quantitated, which is not due to the presence of fetal haemoglobin *per se*, and which disappears physiologically during the first few weeks of life.

Materials and Methods

Adult subjects were healthy blood donors with no clinical disease and a haemoglobin level of at least 13.8 g/100 ml. The *newborns* were babies born in this Hospital without any selection, except for the omission of those who subsequently developed jaundice. Blood samples from *infants* between 1 and 10 weeks of age, and free of any clinical abnormality, were obtained from the welfare and immunization clinic.

Collection of blood specimens. Venous blood from adults and cord blood from newborns was collected with one-fifth volume of ACD solution (disodium citrate, monohydrate, 2 g, glucose, 3 g, in a final volume of 120 ml) and stored for a maximum of 12 h at 4°C. Blood from infants was obtained by heelprick and collected directly in 15 volumes of barbitone buffered saline (2.5 mM barbitone-HCl, pH 7.4, in 0.147 M NaCl).

Osmotic fragility curves or 'fragiligrams' were obtained as described by Danon [2] by use of a fragiligraph, model D2 (Elmedix, Haifa, Israel). When ready for the test, each sample

was centrifuged, the packed cells were diluted 1:50 with buffered saline and equilibrated for 30 min. Exactly 40 µl were then delivered in the dialysis frame by using a 50 µl precision syringe (model 705 Hamilton, Whittier, CA). The test was then carried out as recommended in the manufacturer's handbook.

In order to ensure comparability of results to each other and to those obtained by other methods we always referred to the salt concentration curve, with the following additional precautions: 1) The water chamber of the instrument was always kept at a temperature of 30°C by circulation of thermostated water through the jacket; the water required to fill the chamber was pre-equilibrated at the same temperature. 2) The salt calibration was carried out daily. 3) In every fragiligram the cumulative and the salt curve were recorded simultaneously. In a perfect test the salt curve should tend asymptotically to 90% of the full-scale deflection. All fragiligrams in which the end value of the salt curve was outside the range 85–95 were rejected (in order to achieve this, it was sometimes necessary to repeat a fragiligram up to three times). (4) A derivative curve was always recorded simultaneously to a cumulative curve on a sample in which the cumulative and the salt curve had already been recorded (the relationship between derivative and salt curve could then be obtained by reference to the cumulative curve present in both tests). When all these precautions are taken it is found that multiple curves on the same sample are always closely superimposable. By contrast, comparison of cumulative curves recorded without simultaneous salt curves was unsatisfactory. We have verified that under the conditions described above venous blood and capillary blood yield the same results, and that short term storage in ACD has no effect.

Results

Osmotic fragility of red blood cells in newborns compared to adults. We found in many cases that cord blood erythrocytes appear somewhat more resistant than in the adult, in agreement with DANON *et al.* [3]. However, when the difference is expressed quantitatively in terms of mean corpuscular fragility (MCF: the concentration of NaCl at which 50% of red cells are haemolyzed), it is seen not to be statistically significant (table I). Indeed, although the mean value of MCF is slightly lower in newborns, the range of values is so wide (fig. 1), as to overlap almost totally with the values observed in adults (table I, and compare with fig. 1 of FASAN, 4). Thus, the MCF of cord blood is not characteristically different from that of adult blood.

Quantitation of the osmotic fragility range in the erythrocyte population of newborns and adults. One of the advantages of the 'fragiligram' method over the standard technique of testing osmotic fragility is that the former covers continuously the full range of tonicity from that of physiological saline to that of distilled water. Moreover, the instrument can record automatically, in addition to the cumulative osmotic fragility curve, also its first derivative, which gives a direct visual representation of the distribution of red cells having different resistance to lysis within the blood sample. The striking feature of a newborn's

Table I Comparison of three indices of osmotic fragility in erythrocytes from adults and newborns

		MCF[1]		Δ_{20-80}[2]		σ[3]	
		Adults	Newborns	Adults	Newborns	Adults	Newborns
Number tested		130	70	35	35	15	15
Range		0.31–0.52	0.25–0.55	0.07–0.16	0.12–0.20	0.9–1.2	1.3–2.0
Median		0.41	0.40	0.10	0.16	1.1	1.7
Mode		0.42	0.40	0.10	0.18	1.1	1.7
Mean		0.43	0.41	0.10	0.16	1.1	1.67
Standard deviation		0.05	0.04	0.025	0.02	0.096	0.226
Significance of difference between adults and newborns	t	1.62		9.61		8.26	
	P	>0.1		<0.001		<0.001	

[1] Mean Corpuscular Fragility, expressed as the percent NaCl concentration which causes 50% haemolysis

[2] The difference between percent NaCl concentration which causes 20% haemolysis and the concentration which causes 80% haemolysis

[3] The width of the osmotic fragility range within the red cell population, measured as the distance along the abscissa between the peak and the inflection point of the derivative fragility curve

fragiligram, compared to the adult, is the wider range of osmotic fragility (fig 2) This is reflected in a more 'sluggish' sigmoid shape of the cumulative curve, and in a wider base of the bell-shaped derivative curve In order to express quantitatively the amplitude characteristic of a fragiligram, two indices are introduced (1) The difference between the salt concentration which causes 20% haemolysis and the salt concentration which causes 80% haemolysis, which we designate Δ_{20-80} (2) The distance between the peak of the derivative curve and the inflection point of its descending branch, which we designate σ (fig 2), and which is measured along the abscissa in arbitrary units (we have used centimeters for convenience)

The values of Δ_{20-80} in newborns as a group are considerably higher than in adults The difference is highly significant statistically (table I), although there is still ample overlap between the two groups thus, about 30% of the adult samples fall in the cord blood range, and about 50% of the cord bloods fall in the adult range The difference in the values of σ in the two groups is also highly significant statistically moreover, there is no overlap between the two This index effectively

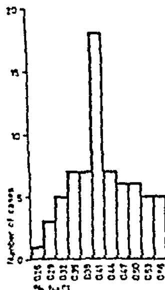


Fig. 1. Distribution of the values of mean corpuscular fragility (MCF) in 70 cord bloods. The cases are grouped according to the NaCl concentration shown on the abscissa, ± 0.01%. Thus, for instance, the cases of the bar marked 0.41 had MCF between 0.40 and 0.42. The NaCl concentrations were read directly from the salt curve on the fragilgram.

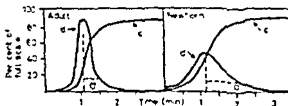


Fig. 2. Typical example of fragilgrams from a normal adult and from a normal newborn. The cumulative curve c) and the derivative curve d) are both shown. The time scale on the abscissa is logarythmically related to decreasing salt concentration. The horizontal broken line joining the peak ordinate of the derivative curve to the inflection point of the latter (σ) is used as a measure of the range of osmotic fragility within the erythrocyte population. The osmotic fragility parameters (see table I) in these two examples were as follows: adult MCF = 0.48, d[illegible] = 0.10, σ = 1.05; newborn MCF = 0.45, d[illegible] = 0.17, σ = 2.0.

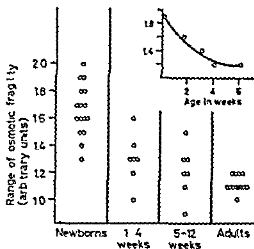


Fig 3 Age-dependence of the osmotic fragility range (scattergram) The values of σ are given on the ordinate The inset shows the time course of change of σ in an individual infant from birth to 6 weeks of age

resolves the two groups, in that σ is always 1 2 or less in adults, but 1 3 or more in newborns (table I)

Age related changes in the osmotic fragility range When does the transition take place from a cord blood type to an adult type of osmotic fragility curve? In order to answer this question we have obtained fragiligrams at serial times from 15 infants between the age of 1 and 12 weeks The transition from one to the other type of fragiligram was found to occur very early in life, usually within 4 weeks (fig 3) This can be shown most clearly by following the changes which take place in an individual baby In the example shown in the inset of figure 3 the value of σ falls exponentially from 1 85 at birth to 1 2 (i e within the adult range) by the age of 6 weeks

Discussion

Erythropoiesis before and after birth is different in several respects and most strikingly in the 'switch' from fetal to adult haemoglobin production Morphologically, erythrocytes in the newborn have a larger size, and a higher haemoglobin content than in the adult, while the haemoglobin concentration is similar to that in the adult [7] Metabolically, differences have been reported in many of the red cell

enzymes [15, 21]. Functionally, it has been proven that at least some of the newborn's erythrocytes have a shorter life span than in the adult [8, 13], but the mechanism for this is unknown.

The osmotic fragility of red cells is a composite index of their shape, hydration and, with certain limitations, of their proneness to in vivo destruction. Indeed, whereas high osmotic resistance does not always correlate with high viability (the typical contradictory example being thalassaemia major), low osmotic resistance invariably indicates that the cells tend to a spherical shape, and therefore are liable to premature removal from the circulation. On the average, the osmotic fragility of erythrocytes is similar in newborns and adults. But this current statement [17, 18] must be qualified by reference to previous work which had already demonstrated the simultaneous presence of unusually resistant and unusually fragile red cells in cord blood [e.g. 20]. WAUGH *et al.* [19: previous papers are quoted in their comprehensive article] even coined the term 'anisohaemolysis' for this phenomenon.

We have confirmed these earlier observations, as well as more recent ones [3, 16]. The MCF values are not significantly different in newborns and adults (table I) and are in good agreement with those reported by CRAWFORD *et al.* [1] and by HENDRICKSE and WATSON-WILLIAMS [7a] in Nigeria. On the other hand, comparison of full fragility curves (fig. 2) clearly demonstrates that the span of haemolysis from higher to lower salt concentrations is wider in newborns than in adults. In addition, exploiting the greater accuracy afforded by the automatic over the manual technique, we were able to obtain a quantitative expression of the degree of anisohaemolysis, by the two parameters which we have called Δ_{10-90} and σ[1] respectively.

It has been found in earlier [illegible]

[illegible]

[illegible]

indicate that also in man haemoglobin F is not directly responsible for the characteristic features of the newborn's fragiligram (high values of Δ_{10-90} and of σ). a) The time course of 'conversion' from neonatal to adult type of fragiligram is quite different from the time course of

[1] It may be noted that in order to determine σ it is necessary to obtain a derivative curve and to locate its inflection point. This was done by eye in our study, and estimates by independent observers agreed within less than 10%. By contrast, the determination of Δ_{10-90} does not require any subjective evaluation and can be done even on data obtained from standard (non-automatic) osmotic fragility curves.

Table II Osmotic fragility in subjects with hereditary persistence of fetal haemoglobin

Case	Age years	Haemoglobin electrophoresis	Percentage of Hb F	Fragilgram data		
				MCF	Δ_{10-90}	σ
1	5	A+F	30	0 44	0 09	1 0
2	4	S+F	48	0 38	0 12	1.2
3	7	A+F	35	0 4?	0 11	1 1

The diagnosis of hereditary persistence of haemoglobin F was based on the following findings (in addition to those shown in the table) a) There was uniform distribution of haemoglobin F within the red cell population (as judged by the elution technique 10] b) There was no clinical or haematological evidence of thalassaemia (nor of sickle cell disease in case 2) c) In cases 1 and 2 the genetic abnormality could be traced to one of the parents

disappearance of haemoglobin F Thus, σ changes abruptly after birth and falls to adult values by 4–6 weeks (fig 3), at a time when haemoglobin F is still the major haemoglobin component in the infant's blood (fetal haemoglobin decreases to adult range only at the age of 4–8 months [22]) b) In three children heterozygotes for hereditary persistance of haemoglobin F [9] we found that the fragiligram was unquestionably of the adult type (table II) Thus, haemoglobin F is not the basis for anisohaemolysis, i e for the simultaneous presence of unusually resistant and unusually fragile cells

We rather suggest that these two fractions of the erythrocyte population may arise by distinct mechanisms The origin of the more resistant cells has been discussed by DANON *et al* [3], and lies perhaps in 'accelerated erythropoiesis' The origin of the more fragile cells is probably related to one or more of the several metabolic deficiencies of the newborn's erythrocytes [6, 12], which may impinge on membrane function and thus be conducive to spherocytosis The early disappearance of the fragile cells suggests that they are prone to haemolysis *in vivo* as well as *in vitro* We propose to investigate whether this can account for the accelerated erythrocyte destruction that is characteristic of the perinatal period, and whether an exaggerated rate of this process underlies some cases of otherwise unexplained neonatal jaundice

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Summary

The osmotic fragility of red cells was analysed by an automatic technique in 70 newborn babies and in 13 infants who were tested at serial intervals between the age of one and 12 weeks. The most characteristic feature of the newborn's blood is a wide range of osmotic fragility (anisohaemolysis), which can be precisely quantitated by measuring the width of the first derivative of the osmotic fragility curve. The value of this parameter, designated σ, is high in the newborn and usually falls to within the adult range during the first 4–6 weeks of life. These data indicate that fetal haemoglobin (which persists for several months) is not responsible as such for the peculiar osmotic fragility pattern observed in cord blood and that an osmotically fragile fraction of the newborn's red cell population undergoes selective destruction very soon after birth.

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Immunchemische Untersuchungen an Spaltprodukten menschlicher Gammaglobuline

Untersuchungen an Seren mit Hypergammaglobulinämie und Paraproteinämie

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Die in den letzten Jahren gewonnenen Erkenntnisse über die Polypeptidstruktur der Immunglobuline (Ig) von Mensch und Tier, haben mit Bezug auf die Immunglobuline des menschlichen Serums zu folgender Einteilung geführt: Das Immunglobulinsystem besteht insgesamt aus fünf Einzelfraktionen, die nach der Entschließung der World Health Organization des Jahres 1964 [33a] als IgA, IgG und IgM bezeichnet werden; inzwischen sind zwei neue Ig-Klassen nachgewiesen und charakterisiert worden, das IgD und das IgE [26, 27, 33b, 41]. Darüber hinaus wurden in Seren von Gesunden Mikrogammaglobuline (μ-Globuline) aufgedeckt [2], deren Bedeutung als Immunglobuline bisher allerdings nicht geklärt werden konnte. Schließlich wird diskutiert, ob das C-reaktive Protein ebenfalls zu den physiologischen γ-Globulinen gerechnet werden darf [19a, b, 35, 16, 18].

Jedes der fünf erstgenannten Immunglobuline ist durch bestimmte physikalische, biochemische und immunologische Eigenschaften ausgezeichnet. Sie unterscheiden sich also hinsichtlich ihres Molekulargewichtes bzw. ihrer Sedimentationskonstanten, hinsichtlich ihrer elektrophoretischen Beweglichkeit und ihrer Diffusionsfähigkeit sowie ihrer antigenbindenden Eigenschaften. Auf Grund einer Beobachtung von Porter aus dem Jahre 1959 [37] lassen sich die vorgenannten Immunglobuline durch Papain-Einwirkung fermentativ spalten, dabei entstehen klassenspezifische sowie kreuzreagierende Polypeptidketten. Entsprechend ihrer Aminosäurensequenz wurden sie als sogenannte H- (heavy-) und L- (light-) Ketten bezeichnet. Jüngste Forschungsarbeiten haben die Struktur dieser Bruchstücke aufgezeigt.

Es ist heute bekannt, dass an die verschiedenen γ-Globulin-Bruchstücke auch klinisch interessante Eigenschaften, etwa genetische Faktoren, antigenbindende oder Paraprotein-Eigenschaften gebunden sind [1, 6, 8, 9, 10, 11, 12, 13, 16, 18, 28, 38, 39, 40, 52].

In vorliegender Arbeit sollten γ-Globulin-Analysen mit Differenzierung von Papain- und Zysteamin-Spaltprodukten vorgenommen werden, mit der besonderen Fragestellung der Nutzbarkeit solcher Untersuchungen für die klinische Routinediagnostik.

Material und Methodik

Untersucht wurden die Seren von einem Patienten mit chronischer Hepatitis, ferner das Serum eines Kranken mit primär chronischer Polyarthritis, weiterhin das Serum eines Patienten mit chronisch rezidivierender Asthma Bronchitis und schliesslich das Serum eines Patienten mit γG Myelom. Alle Patientenseren waren durch eine Hypergammaglobulinanämie ausgezeichnet.

Die Seren wurden zunächst papierelektrophoretisch und mit der Elektrophorese auf Celluloseacetatfolien (CAF) sowie immunoelektrophoretisch differenziert. Von jedem Serum wurde ferner die Gammaglobulinfraktion durch Rivanolfällung sowie durch Trennung an der DEAE-Säule von den übrigen Serumproteinfraktionen isoliert. Der Globulinkomplex wurde nunmehr jeweils mit Papain, mit Zysteamin als auch mit Papain und Zysteamin behandelt. Die Spaltprodukte wurden immunoelektrophoretisch unter Verwendung eines polyvalenten Antihumanserums sowie monoklonaler Antiseren gegen die verschiedenen Ig Subfraktionen IgA, IgG, IgM, IgD und schliesslich unter Verwendung monoklonaler Antiseren gegen H- und L-Ketten analysiert.

Papierelektrophorese

Die Papierelektrophorese wurde nach dem bekannten Verfahren von GRASSMANN und HANNIG [22, 44] durchgeführt.

Elektrophorese auf Celluloseacetatfolien

Die CAF Elektrophorese wurde im Prinzip nach den Angaben von KOHN [29 a-c] vorgenommen. Zur Auswertung der Farbintensitäten der elektrophoretisch getrennten und mit Amidoschwarz gefärbten Proteinfraktionen wurde der Zeiss-Integralschreiber[1] gewählt.

Immunoelektrophorese

Die Immunoelektrophorese wurde nach den Angaben von GRABAR und WILLIAMS [20, 21] in der Modifikation nach SCHEIDEGGER [42] durchgeführt. Für einzelne Analysensätze wurden darüber hinaus Agarplatten mit den Abmessungen 19 × 12 cm mit 8 Auftragestellen und 7 Antiserumkanälen verwendet.

Als *Antiseren* wurden ein handelsübliches polyvalentes Antihumanserum sowie monoklonale Antiseren gegen IgA, IgG, IgM, IgD[3], aber auch gegen Fab-Fragment (= L-Ketten) und Fc Fragment (= H Ketten)[2] gewählt.

[1] Fa. Zeiss, Oberkochen.

[2] Polyvalentes Antihumanserum sowie Fab- und Fc Fragment Antisera Fa. Hyland Lab., Los Angeles, Calif. USA.

[3] Monoklonale Antisera der Behringwerke, Marburg/Lahn.

Globulin-Darstellung

a) Rivanolfällung. Vorgegangen wurde nach früheren eigenen Erfahrungen [45] bzw. nach den Angaben von Horejsi und Smetana [25]. Handelsübliches Rivanol* wurde als 0,4%ige wässrige Lösung verwendet. 1 ml des zu untersuchenden Serums wurde mit 2,5 ml der Rivanol-Lösung versetzt. Die entstandene Fällung wurde 30 min bei 4500 Upm in der Kühlzentrifuge abzentrifugiert. Der Überstand konnte ohne Dialyse weiter verarbeitet werden.

b) Trennung über die DEAE-Zellulose-Säule [[illegible]]. Angewandt wurde im Prinzip das Ionenaustauscherverfahren nach Peterson und Sober [[illegible]]. Nach Aufbereiten der DEAE-Zellulose und Packen der Säule (2,5 × 40,0 cm, bei Höhe 17,0 cm) wurde die Säule dreimal mit einem Phosphatpuffer (I, pH 7,0) equilibriert. Danach wurde die Säule mit 3,0 ml der Serumprobe überschichtet. Nach erfolgter Aufnahme des Serums von der Säule wurde mit einem anderen Phosphatpuffer (II, pH 6,[illegible]) die Säule aufgefüllt. Dies wurde so oft wiederholt, bis die erste γ-Globulinfraktion eluiert war. Eine auf gleiche Weise gewonnene zweite Fraktion enthielt γ- und β-Globulin-Komponenten.

Puffer I: 0,01[illegible] m, pH 7,0: 1000,0 ml NaH_2PO_4, 8,5 ml Na_2HPO_4

Puffer II: 0,0175 m, pH 6,5: 1000,0 ml NaH_2PO_4, 2,6 ml Na_2HPO_4

Papainisierung

Die beiden γ-Globulinproben, die nach Rivanolfällung sowie die nach Trennung an der DEAE-Säule jedes Serums wurden den nachfolgenden Behandlungen unterzogen. Jede Probe wurde zunächst mit Papain versetzt, wobei im Prinzip nach den Angaben von Porter [37, 38] vorgegangen wurde. Nach einer Gesamteiweißbestimmung mit dem Autoanalyser wurden die Proben mit Papain und einem Puffer versetzt. Das Mengenverhältnis betrug 1 mg Papain auf umgerechnet 100 mg Eiweiß. Der Puffer bestand aus 0,1 m Na-Phosphatpuffer pH 7,0, 0,01 m Cystein und 0,001 m Äthylendiamintetraacetat. Dieses Gemisch wurde bei 37 °C für die Dauer von 3 h inkubiert. Anschließend wurden die einzelnen Proben 4 h lang gegen 100 ml eines 0,01 m Phosphatpuffers pH 7,6 und danach gegen 1000 ml desselben Puffers 24 h dialysiert.

Behandlung der Proben mit Zysteamin

Im Hinblick auf das bei Deutsch und Morton [7, 8, 33] beschriebene Verfahren wurde folgendermaßen vorgegangen: Eine 1 m Zysteaminlösung in Sörensen-Puffer pH 7,2 und 0,0[illegible] m wurde jeweils 1:10 mit der γ-Globulin-Probe verdünnt. Das Gemisch wurde [illegible] h bei Raumtemperatur inkubiert, anschließend 2 h dialysiert gegen 0,0[illegible] m Monojodazetat.

Resultate

Befunde bei Aufbereitung bzw. γ-Globulingewinnung der Patientenseren. Bei Vergleich der γ-Globulin-Präparate nach Rivanolfällung sowie nach Trennung an der DEAE-Säule, ist zunächst festzustellen, dass beide Methoden eine relativ gute Ausbeute hinsichtlich der γ-Globulinfraktion ergaben. Bei beiden Methoden waren allerdings auch Verunreinigungen durch ein bis zwei Komponenten des β-Bereiches und in Einzelfällen sogar durch α_2-Globulinfraktionen nicht zu vermeiden. In Fällen mit relativ hoher γ-Globulin-Konzentration, z. B. beim γ-G-Myelom, aber auch bei der chronischen Hepatitis, war der Reinheits

* Rivanol Hoechst DAB 6

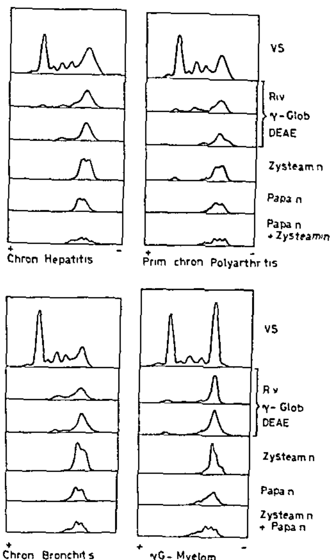


Abb 1 Diagramme der elektrophoretischen Analysen auf Zelluloseazetatfolien der untersuchten Seren und deren γ Globulinkomplexe

grad der γ-Globulin-Fraktion wesentlich hoher als im Falle etwa der primär chronischen Polyarthritis Diese Feststellungen waren sowohl mit der Immunoelektrophorese als auch mit den relativ groben Methoden der Papier- und CAF-Elektrophorese zu treffen (Abb 1)

Befunde nach Spaltung der γ-Globulinfraktion durch Papaineinwirkung Bei allen untersuchten Seren gelang die Darstellung von zwei Komponenten, die sich sowohl in ihrer elektrophoretischen Beweglichkeit als

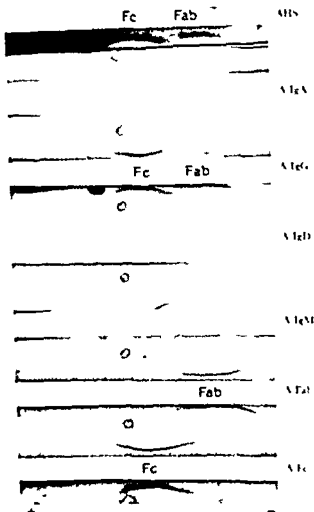


Abb. 2. Immunelektrophoretische Analysen des γ-G-Globulins des Serums einer chronischen Hepatitis nach Behandlung mit Papain

auch in ihren immunologischen Eigenschaften (Kreuzungsphänomen) voneinander unterschieden. Sie entsprachen, wie mit monoklonalen Antiseren bewiesen werden konnte, den Fc- (H-chains) bzw. den Fab- (L-chains) Fragmenten (Abb. 2). Im Falle des hier analysierten γ-G

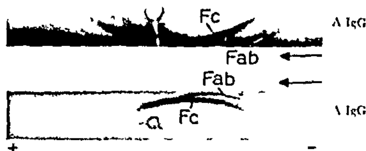


Abb 3 Immunoelektrophoretische Befunde der γ-G Myelom Fraktion Oben nach Behandlung mit Papain unten nach Einwirkung von Papain und Zysteamin

Myeloms konnte zwischen dem typischen Fc-Fragment und dem ebenfalls charakteristischen Fab-Fragment eine zusätzliche Komponente wahrgenommen werden, die sowohl das Fc- als auch das Fab-Präzipitat total kreuzte (Abb 3) Mit der CAF-Elektrophorese war das γ-Globulin nach Papaineinwirkung als ein relativ breiter Komplex darstellbar, der seinerseits zwei bis drei Subfraktionen erkennen liess Eine Differenzierung in γ-Globulin Komponenten mit exakt definierbaren elektrophoretischen Beweglichkeiten gelang nicht (Abb 1)

Im übrigen ist bemerkenswert, dass die vergleichsweise mit angesetzten, nicht gespaltenen Vollseren bei Verwendung monoklonaler Antiseren ebenfalls eine Differenzierung in H- und L-Ketten zulassen, wobei lediglich die elektrophoretischen Beweglichkeitsunterschiede der Fc- und Fab-Fragmente nicht so deutlich in Erscheinung treten Sie sind aber prinzipiell nachweisbar (Abb 4)

Befunde nach Spaltung der γ Globulinfraktion durch Zysteamineinwirkung Hier konnte in den Fällen mit chronischer Hepatitis sowie der primär chronischen Polyarthritis ebenfalls ein Spaltungsphänomen mit der Darstellung von zwei Komponenten mit unterschiedlicher Wanderungsgeschwindigkeit festgestellt werden, wobei die beiden Komponenten keine sichere Kreuzung, sondern nur eine Teilidentität aufwiesen Sie entsprachen aber wiederum den H- und L-Ketten der γ-G Fraktion, wie dies unter Verwendung monoklonaler Antiseren gezeigt werden konnte (Abb 5)

Bei dem Serum mit γ G-Myelom konnte eine sichere Aufspaltung nicht erzielt werden Stattdessen zeigte sich eine Doppelung der Präzipitationslinien des γ-G Globulins, die im gleichen Masse mit dem polyvalenten Antiserum, dem anti-γ-G-Serum sowie mit monoklonalen Antiseren gegen H-, nicht aber gegen L-Ketten auspräzipitierte

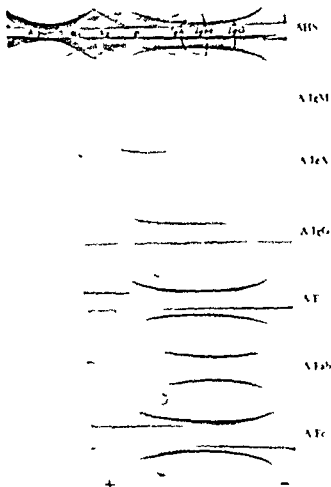


Abb. 4 Immunelektrophoretischer Analysensatz des unbehandelten (Voll-)Serums des Patienten mit chronischer Hepatitis unter Verwendung eines polyvalenten Antihumanserums (AHS) sowie verschiedener monovalenter Antiseren gegen die bekannten Immunglobulinklassen und gegen H- und L-Ketten von IgG

Befunde nach Spaltung der γ-Globulinfraktion durch Papain und Zysteamineinwirkung. Die Ergebnisse dieser Versuchsreihe entsprachen im wesentlichen den Befunden allein nach Papaineinwirkung, d. h. es zeigte sich in allen Fällen eine Aufspaltung des γ-Globulin-Komplexes in

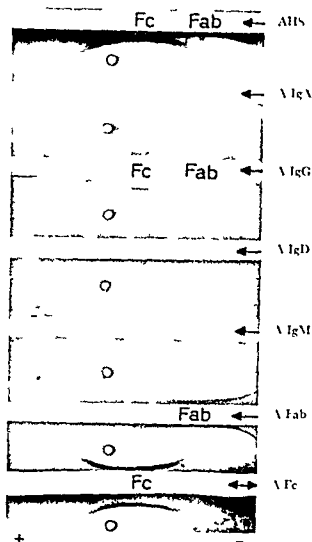


Abb 5 Immunoelektrophoretischer Analysensatz des γ G Globulins des Serums einer chronischen Hepatitis nach Behandlung mit Zysteamin

zwei Komponenten Die Fraktion näher der Auftragestelle entsprach den H-Ketten, die näher der Kathode gelegene den L-Ketten Es ergab sich jedoch mit Regelmässigkeit ein auffälliges Phänomen, nämlich die Feststellung, dass bei Reaktion des gespaltenen Komplexes gegen das monovalente γ-G-Globulin jeweils nur die Komponente der H-Ketten zur Darstellung gelangte, nicht aber die der L-Ketten (Abb 6)

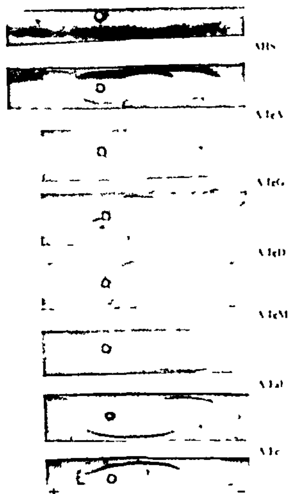


Abb. 6. Immunelektrophoretischer Analysensatz des γ G-Globulins des Serums einer chronischen Hepatitis nach Behandlung mit Papain und Zystein

Diskussion

Die Befunde der vorliegenden Serumanalysen nach Papain-Einwirkung, mit der Darstellung von zwei Komponenten mit unterschiedlichen elektrophoretischen und immunologischen Eigenschaften, wie sie regelmässig in den vorgegebenen Globulin-Komplexen nachzu

weisen waren, bestätigen die seit Porter [37] erzielten gleichartigen Ergebnisse [1, 3, 5, 9, 10, 13, 14, 18, 52, 54]. *Bekanntlich* entspricht die eine der beiden Komponenten, und zwar die der Kathode näher gelegene, dem sogenannten Fab-Fragment, das seinerseits zur Hauptsache aus L-Ketten und zu einem kleinen Teil aus H-Ketten besteht [14, 15, 54]. Die andere Komponente, die der Auftragestelle näher gelegen ist, entspricht dem sogenannten Fc-Fragment, das ausschliesslich aus H-Ketten besteht.

Die ersten Untersuchungen über derartige, durch fermentative Aufschliessung von γ-Globulinen gewonnenen Spaltprodukten, sind erstmals von Porter [37] an Seren immunisierter Tiere vorgenommen worden, ohne dass damals die Bedeutung dieser Bruchstücke mit Bezug auf eine Spezifizierung der γ-Globulinfraktionen in verschiedene Immunglobulin-Klassen schon klar erkannt worden wäre. Dank der in den letzten Jahren erfolgten chemischen und immunchemischen Aufklärungsarbeit an diesen Spaltprodukten [5, 6, 10, 14, 15, 16, 32, 38, 39, 40], wissen wir heute, dass jedes Immunglobulin-Molekül aus zwei klassenspezifischen H-Ketten und zwei L-Ketten bestimmter Prägung (K=I oder L=II) besteht [9, 10, 13, 14, 15, 16, 28, 33, 39, 47, 54]. Dabei sind die L-Ketten allen Immunglobulinen gemeinsam, zumindest untereinander immunologisch verwandt, während die H-Ketten durch die klassenspezifischen Merkmale α (IgA), γ (IgG), μ (IgM), δ (IgD) und ε (IgE) ausgezeichnet sind. Die L-Ketten kommen in zwei verschiedenen Typen (I oder II, s. o.) vor, wobei jedes Im*munglobulin-Molekül entweder zwei L-Typ I-Ketten oder zwei L-Typ* II-Ketten besitzt. In einem Individuum lassen sich aber sowohl Moleküle mit L-Ketten vom Typ I als auch Moleküle mit L-Ketten vom Typ II nachweisen, wobei das Verhältnis von Typ I zu Typ II von Immunglobulinklasse zu Immunglobulinklasse unterschiedlich angegeben wird [in 47, in 54].

Diese Kenntnisse über Struktur und Funktion der Immunglobuline haben zu völlig neuen Aspekten auch in der klinischen Medizin geführt. Sie erlangen insbesondere Bedeutung bei der Aufklärung von Anomalien im Bereich der γ-Globuline bzw. für die Eruierung von Strukturänderungen innerhalb eines γ-Globulin-Moleküls bei Paraproteinosen [11, 18, 23]. Unter der Voraussetzung, dass in Zukunft spezifische monovalente Antiseren gegen H- und L-Ketten zur Verfügung stehen werden, und dass unter Umständen der methodische *Aufwand des fermentativen Spaltungsprozesses auf klinische Routine-*

Ansprüche abgestimmt oder sogar entbehrlich werden kann, stellt diese γ-Globulin-Analyse eine wesentliche Bereicherung für die Paraprotein- und wahrscheinlich auch für die allgemeine γ-Globulin-Diagnostik pathologischer Seren dar.

Inzwischen gesammelte Erfahrungen und weitere Erkenntnisse an pathologischen menschlichen Seren, wie sie das Schrifttum aufweisen, haben gezeigt, dass sowohl bei den Paraproteinen als auch bei den stark vermehrten γ-Globulinen im Rahmen anderer Erkrankungen (Hepatitis, primär chronische Polyarthritis, Retikulosen und andere maligne Systemerkrankungen) die meisten Strukturveränderungen an die H-Ketten gebunden sind [1, 3, 43, 52]. Bei Paraproteinen finden sich Anomalien aber an H- und L-Ketten [43]. Darüber hinaus wurden in den letzten Jahren Krankheitsfälle beschrieben, welche durch ein Syndrom mit Paraproteinämie, Paraproteinurie und Hepatosplenomegalie gekennzeichnet sind [17, 34]. Auf Grund von Strukturanalysen dieser Paraproteine durch FRANKLIN [17] konnte gezeigt werden, dass bei dieser Paraproteinämie die atypische Eiweissfraktion nur aus strukturveränderten H-chains aufgebaut ist. Nach seinem Entdecker wird dieses Syndrom seither Franklin-Syndrom, die Krankheit H-Ketten-Krankheit genannt. Gewissermassen das Pendant zur «H-chain-disease» stellt die Bence-Jones-Paraproteinämie mit Paraproteinurie als klinisches Syndrom dar. Hier bestehen die Paraproteinmoleküle nur aus L-Ketten.

Nach heutiger Kenntnis ist die γ-Globulin-Synthese der verschiedenen Immunglobulin-Klassen an ganz bestimmte Zellklone gebunden [4]. Möglicherweise erfolgt sogar die Polypeptidketten-Bildung klonal uns somit isoliert, so dass H- und L-Ketten erst sekundär zu klassenspezifischen Ig-Systemen komplettiert werden müssen.

In vorliegender Arbeit standen die vorgenannten monovalenten Antiseren gegen die verschiedenen Ig-Klassen sowie gegen H-Ketten von IgG und gegen L- (Typ I und Typ II) -Ketten von Bence-Jones-Proteinen zur Verfügung. Die darstellbaren Bruchstücke nach Papain- und Zysteamin-Einwirkung konnten damit exakt identifiziert werden. Erwähnenswert erscheint ein Befund bei Analyse des γ-G-Myelomproteins, wobei eine zusätzliche Präzipitationslinie mit – wenn auch geringer, aber dennoch definierbarer – unterschiedlicher elektrophoretischer Beweglichkeit zwischen Fab- und Fc-Fragment wahrgenommen werden konnte. Ähnliche Beobachtungen wurden an den Immunglobulinen von Versuchstieren gemacht. Eine genaue Zuordnung

des in der vorliegenden Analysenreihe beobachteten Präzipitationsphänomens zu einer immunchemisch definierbaren γ-Molekül-Komponente gelang nicht. Möglicherweise handelt es sich um eine Variante des Fd-Fragments. Nachdem in den übrigen untersuchten pathologischen Seren eine derartige Fraktion nicht nachgewiesen werden konnte, darf angenommen werden, dass es sich um eine Besonderheit des Paraproteins handelt.

Die mit Zysteamin behandelten gleichen Seren, deren Spaltprodukte prinzipiell anderer Art sein sollten, als diejenigen nach Papainspaltung, ergaben überraschenderweise «phänomenologisch» ähnliche Präzipitate, d.h. eine schneller wandernde und eine näher der Kathode zugewandte Linie, wie das auch bei Papainspaltung wahrgenommen werden konnte. Mit einem polyvalenten Antiserum stellten sich allerdings die beiden Präzipitate nicht mit einem Kreuzungsphänomen dar, sondern zeigten Teilidentität. Diese Befunde bestätigen Beobachtungen, wie sie auch von anderer Seite [28, 30, in 47] mitgeteilt wurden.

Die Feststellung schliesslich, dass auch an nicht gespaltenen γ-Globulinen bzw. an unbehandelten Vollseren unter Verwendung monoklonaler Antiseren, eine Differenzierung des Ig-Systems und darüber hinaus sogar der isolierte Nachweis von H- und L-Ketten möglich ist, dürfte für die zukünftigen Routine-Untersuchungen an γ-Globulinen nicht nur im Forschungslaboratorium sondern auch für das klinische Labor eine wertvolle Bereicherung darstellen. Für die monoklonalen Ig-Fraktionen ist dies bereits eine geläufige Methode zur Differenzierung der γ-Globuline geworden. [43] Mit immunchemischer Technik gelingt es darüber hinaus, die einzelnen Globulinfraktionen *quantitativ* zu bestimmen [12, 31, 19, 51]. Es wird in naher Zukunft möglich sein, auch H- und L-Ketten-Relationen quantitativ zu erfassen.

Über die diagnostisch verwertbare Anwendung monoklonaler Antiseren gegen H- und L-Ketten liegen bislang allerdings nicht genügend praktische Erfahrungen vor. Eigene Untersuchungen an insgesamt 160 pathologischen Seren mit γ-Globulin-Varianten lassen eine sichere Differenzierung in H-, L- und Bence-Jones-Typen auch am nicht gespaltenen γ-Globulin zu [43].

Prinzipiell konnten die vorliegenden Analysen und deren Befunde zeigen, dass γ-Globuline durch Einwirkung von Papain bzw. Zysteamin, ungeachtet ihrer krankheitsspezifisch verursachten Anreicherung, grundsätzlich gleichartige Spaltprodukte aufweisen, was auf einen

gleichartigen Bauplan der pathologischen wie der physiologischen γ-Globuline schliessen lässt. Paraproteine zeigen dabei gewisse Strukturänderungen, die offenbar an den H-Ketten fassbar werden.

Zusammenfassung

Die γG-Globuline der Seren von 8 Patienten mit verschiedenen Erkrankungen (chronische Hepatitis, primär chronische Polyarthritis, chronisch rezidivierende [illegible] [illegible]) wurden durch fermentative Spaltung mit Papain sowie Behandlung mit Zystein und nachfolgende immunologische Differenzierung analysiert. Verwendet wurden monoklonale Antiseren gegen die gereinigten Seren zur vergleichenden Beurteilung der Spaltprodukte der verschiedenen γG-Globuline. In jedem Fall war eine Spaltung in H- und L-Ketten möglich. Bei einem γ-Myelom war das spezifische Paraprotein-Präparat an die H-Ketten gebunden. Es war ausserdem ein zusätzliches H-Ketten-spezifisches Präparat nachweisbar. Die Differenzierung in H- und L-Ketten ist sowohl am gespaltenen γG-Globulin als auch am nicht behandelten Serum möglich.

Summary

The γG-globulins of sera of 8 patients with different diseases (chronic hepatitis, primary chronic rheumatoid arthritis, chronic [illegible] [illegible] a γG-myeloma) but all with [illegible] gammaglobulinemia have been analyzed by enzymatic cleavage by papain as well as by cysteine and immunochemical differentiation using monoclonal antisera against polypeptide chains for comparing the γ-globulin split products. A cleavage into H- and L-chains was possible in every case. In one γG-myeloma the paraprotein preparate was [illegible] to the H-chains. Moreover in this case of paraproteinemia another H-chain specific [illegible] could be demonstrated. H- and L-chains could be demonstrated in the cleaved gamma globulin preparate as well as in the not treated patients' sera using monoclonal antisera against H- and L-chains.

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Binding of Folic Acid Activity by Body Fluids

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Total folic acid activity (FAA) in most body fluids has been studied in detail, and the influence of various pathological conditions on it is fairly well known. The question of whether, and to what extent, FAA is bound to proteins or naturally present in a free state has been basely studied. As early as 1956, Toennis *et al.* [15] noticed this activity in Cohn's fractions IV+V and certain sub-fractions. Condit and Grob [1] noted in electrophoresis a kind of overlapping between the albumin spot and FAA but interpreted it as methodical. Johns *et al.* [7] assumed that there was little if any free FAA in fasting plasma. Neal and Williams [11] experimented with the behaviour of labelled FAA in electrophoresis and found that it moved faster towards the anode than did albumin. Only slight activities were observed in the protein region. The majority of these coincided with albumin. In addition, Hampers *et al.* [4], Sevitt and Hoffbrand [13], and Retief and Huskisson [12] have discussed the question of the binding of FAA in their papers on renal and hepatic diseases. Metz and Herbert [10] mentioned unsuccessful attempts to demonstrate binders for folic acid in normal human serum, analogous to those present for vitamin B_{12}. Metz *et al.* [9] found with labelled FAA, however, that binding, though weak, does occur, and that FAA can be eliminated from the serum by coated charcoal.

The FAA of milk has been found to be bound by proteins. The binding seems to be highly specific, as it is not broken by autoclaving [2, 3]. Similar results for milk were reported by Metz and Herbert [10], and Metz *et al.* [9].

In the present study the behaviour of FAA vis-à-vis protein fractions

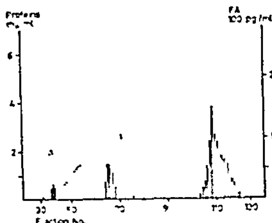


Fig. 1. The serum of a young healthy male test subject (FAA 5.2 ng ml) fractioned with Sephadex® G-200. The dots indicate protein distribution, the vertical lines the FAA distribution.

in Sephadex® filtrations was observed. Sera, cerebrospinal fluids, mother's milk and synovial fluids were filtered.

Material and Methods

Venous blood was taken into a 100 ml centrifuge tube from male and female patients [illegible] study were carried out without any preserving agents. The sera were filtered while they were fresh, and the technique described in connection with the preliminary studies [8] was followed in every detail.

The cerebrospinal fluids were drawn from healthy volunteers by lumbar puncture. The specimens were immediately centrifuged and placed in gel filtration. The technique described above was used.

The mother's milk specimens also were centrifuged while they were fresh, and the skimmed milk was then filtered. The technique used was as described above.

The synovial fluid was also centrifuged in order to eliminate whatever cell contamination may have been present. No preservatives were added either here or in the cerebrospinal fluid and milk specimens. The technique of filtration was as described above.

The filtration gels were Sephadex® G-200 and G-25 (fig. 2). The FAA activity of the fractions obtained was measured by the Lucaset method as described earlier [8]. The protein determinations were carried out with the biuret reaction [8].

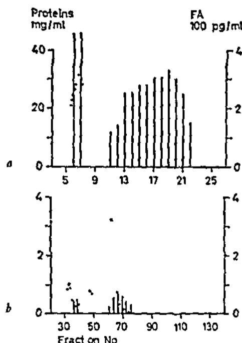


Fig. 2a. The same serum as in figure 1. Filtration with Sephadex® G-25. *b* The protein zone FAA obtained on the previous filtration re-filtered with Sephadex® G-200. For explanations see figure 1.

Results

Figure 1 shows the typical form of FAA distribution in the Sephadex® G 200 filtration of the serum of a healthy subject. FAA is noted in 3 maxima: first maximum (5%) in the zone of high-molecular globulins, second maximum (30%) in the albumin zone, and the remainder, third maximum (65%) in the post-protein zone. When the serum of the same person was filtered with Sephadex® G-25 (fig. 2a) and subsequently the FAA of the protein zone was re-separated with Sephadex® G-200 (fig. 2b), it was found that no L. casei activity was separated from the FAA of the protein zone on re-filtration. The G-25 filtration had separated the free (or liberated) FAA. In the re-filtration of the FAA of the protein zone the recovery had been complete. This activity was clearly divided into two maxima, which coincided completely with the maxima of figure 1.

Figure 3a shows the distribution of FAA in filtered cerebrospinal fluid: only 5% is in the protein zone and the remaining 95% in the

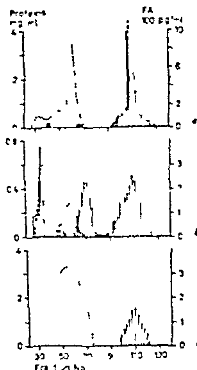


Fig. 3a. Cerebrospinal fluid of a healthy subject (FAA 14.5 ng ml in Sephadex® G-200 filtration. *b* Skimmed mother's milk (FAA 6.6 ng ml in Sephadex® G-200 filtration. *c* Synovial fluid (FAA 3.3 ng ml in Sephadex® G-200 filtration. For explanations see figure 1

post protein zone A small activity peak is visible in the pre albumin area and a slightly more pronounced activity in the posterior margin of the albumin

Figure 3b shows the distribution of FAA in filtered fresh mother's milk (skimmed) It may be pointed out, however, that in skimmed milk the FAA was 6.6 ng/ml and in the creamy component 185.5 ng/ml This latter part has so far not been fractioned and its FAA reviewed The measured protein zone covered 60% of all the FAA obtained, the balance being in the post protein zone The two maxima of the protein zone were almost equal in size, about 30% of the FAA obtained Consequently, the percentage of FAA in the protein area is higher in skimmed milk than in serum

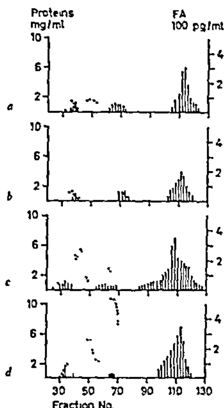


Fig 4 Sera of patients with various diseases in Sephadex® G-200 filtration *a* Pernicious anaemia (FAA 4 0 ng/ml), *b* megaloblastic tapeworm anaemia (FAA 5 6 ng/ml), *c* myeloma (FAA 5 6 ng/ml), *d* folate deficiency (FAA 2.5 ng/ml) For explanations see figure 1

Figure 3c reveals that the FAA of the synovial fluid, studied by this method, remained entirely outside the protein zone

Figure 4 presents the results from pathological sera (a) in pernicious anaemia (untreated) 14% (4+10%) of the FAA was in the protein zone, (b) in megaloblastic tapeworm anaemia (untreated) 16% (3+13%), (c) in myeloma 11% (4+7%), and (d) in folic acid deficiency originating in the small intestine 10% (7+3%)

Discussion

The results of this study permit the conclusion that serum, examined by this method, contains natural FAA even in the protein zone (approximately one-third), although the majority (approximately

two-thirds) emerge beyond the protein fractions The present experiments, however, failed to show whether the 'free' FAA emerging in the post protein zone had during filtration totally or partially separated from the FAA in the protein zone Furthermore, the chemical structure and molecular size of the FAA in the various fractions are not yet clear In preliminary experiments it was found [8] that about one third of the FAA of the post protein zone was dialysable through a membrane which passes particles of a molecular weight of 5000 It is possible that polyglutamates of chains of varying lengths are involved

The Sephadex® gel filtration distinctly separates the FAA that remains in the protein zone When first filtered with Sephadex® G-25 the above 'free' FAA fraction is distinctly separated from the 'bound FAA fraction No further activity appears to be separated from the latter during the period of re fractionation with Sephadex® G-200 This may mean that the binding of FAA by various proteins in this zone is relatively firm From this two activity maxima are clearly distinguishable on the basis of the preliminary immunoelectrophoretic studies the first maximum contained very high molecular globulins, the second proteins equal to the size classes of albumin and transferrin Three main lines require further study the exact definitions of the proteins which carry the FAA in the protein zone, the additional capacity, if any, of these proteins to bind FAA, and the role of these proteins and their affinity to FAA in folate metabolism in physiological and pathophysiological conditions Although the FAA of the protein zone in the present patients with megaloblastic anaemia was considerably smaller than in healthy subjects, no conclusions can be drawn on the basis of these few cases The 'bound' volume of FAA of a patient with folate deficiency is also exceedingly small In myeloma the relative shares of FAA do not seem to differ distinctly from normal On the basis of experience obtained in our laboratory to date it would seem that in older age groups especially women, the first FAA maximum is often weak or perhaps absent

The maxima recorded for mother's milk were largely similar to those obtained for sera, though they are considerably more pronounced than in the sera, and distinctly more FAA is verified in the protein zone (a higher degree of binding?) than for serum The present results are parallel with those recorded by Ghitis *et al* [3], according to whom milk evidently had at least two fractions which may bind FAA firmly

Closer study, in particular, is required to determine the fraction to which the high FAA's in the creamy component of the milk are bound.

According to earlier studies, the FAA of the cerebrospinal fluid is of the same or even greater magnitude than the corresponding serum activity [5, 6, 14, 16]. According to the present findings, practically all the FAA of the cerebrospinal fluid is in the 'free' form. Only trace spots are noted in the protein zone. From the neurological point of view, it would apparently be interesting to study the possible effects of various pathological conditions of the central nervous system on the binding of cerebrospinal fluid FAA. To this end, both the total FAA and its distribution into the various fractions of cerebrospinal fluids should be determined.

Summary

The presence of natural FAA sera, cerebrospinal fluid, mother's milk and synovial fluid was studied. In the serum of healthy subjects the FAA was divided into 3 maxima (1) in the high molecular (globulin) zone, (2) in the albumin zone, (3) in the post-protein zone. Sera of patients with pernicious anaemia, megaloblastic tapeworm anaemia, folate deficiency and myeloma showed the same FAA maxima. The FAA of cerebrospinal fluid was almost entirely located in the post protein zone. In skimmed mother's milk, the FAA maxima were located on the same sites inside and outside the protein fractions. The FAA of the synovial fluid fell completely outside the protein zone.

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Vergleichende Untersuchungen zur Erythrozytenkinetik und zur Knochenmarksmorphologie bei Patienten mit Osteomyelosklerose und Osteomyelofibrose

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Die Osteomyelosklerose bzw. die Myelofibrose sind heute durch die breite Anwendung der intravitalen Knochenbiopsie häufig diagnostizierte Krankheitsbilder [1, 4, 5, 11, 21, 27, 33]. Die bei derartigen Patienten frühzeitig auftretende therapieresistente Anämie stellt den behandelnden Arzt vor viele diagnostische und therapeutische Probleme [9, 12, 28, 33]. Durch die Ergebnisse moderner nuklearmedizinischer Untersuchungsverfahren unter Verwendung der Nuklide ^{59}Fe und ^{51}Cr wird heute die Ursache dieser Anämie vorwiegend in der Kombination einer Erythrozytenminderproduktion und eines gesteigerten peripheren Abbaus der roten Blutkörperchen gesehen [2, 3, 8, 17, 20, 24, 30, 34]. Der Leber, vor allem aber der Milz kommt in der Anämiepathogenese häufig eine Doppelrolle zu, da sie als extramedulläre Blutbildungsstätten einmal wesentlich zur Produktion der roten Blutkörperchen beitragen, andererseits aber in diesen Organen oft eine gesteigerte Erythrozytoklasie auftritt [2, 16, 17, 30]. Auch die Ausbildung eines vergrösserten Erythrozytendepots in der oft stark vergrösserten Milz im Sinne eines Hyperspleniesyndroms ist häufig von entscheidender Bedeutung für die Entwicklung des Erythrozytendefizits [13, 31]. Wie Hittmair [12] erst 1969 betonte, sind vergleichende Darstellungen zwischen den Untersuchungsergebnissen zur Erythrozytenkinetik und morphologischen Knochenmarksbefunden sowie klinischen Parametern bis jetzt überhaupt nicht oder nur in sehr geringem Umfang durchgeführt worden, die bei der meist nur sehr

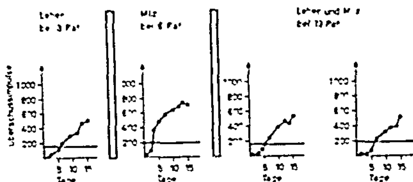


Abb. 1. Ort des maximalen Erythrocytenabbaus bei Patienten mit Myelofibrose und Osteomyelosklerose

kleinen Zahl von untersuchten Kranken keine bindenden Aussagen gestatten.

Material und Methoden

Bei 34 Patienten mit Osteomyelosklerose bzw. Myelofibrose erfolgte die Bestimmung der «scheinbaren halben Erythrocytenüberlebensdauer» nach den Angaben von Mollison und Veall [19, 33] bei gleichzeitiger Messung von Oberflächenaktivitäten und Berechnung sog. «Überschußimpulse» bzw. «zusätzlicher Aktivitätswerte» nach dem Verfahren von Huguhs-Jones und Szur [15]. Bei 16 Kranken wurden Untersuchungen zur Eisenkinetik nach den Angaben von Huff et al. [14] bzw. Pollycove und Mortimer [29] bei gleichzeitigen Oberflächenaktivitätsmessungen über Leber, Milz, Herz und Kreuzbein durchgeführt.

Die Diagnose einer Osteomyelosklerose bzw. Myelofibrose wurde stets durch die histologische Untersuchung eines mittels intravitaler Knochenbiopsie [1] gewonnenen Knochenzylinders gesichert. Die Krankheitsstadien der Osteomyelosklerose wurden nach den Angaben von Oechslin [23] in 3 Phasen differenziert: 1. Die fibroosteoklastische Phase oder Stadium I, 2. die osteoidoplastische Zwischenphase oder Stadium II und 3. die osteoplastische Stabilisierungsphase oder Stadium III.

gegangen werden.

Wir sind uns bewusst, dass gelegentliche Phasen zwischen den einzelnen Krankheitsstadien erfasst werden, die nur bedingt eine Zuordnung in die angegebene Stadieneinteilung gestatten. Wegen der für die Vergleichsdarstellungen besseren Übersicht wurde nach mehreren Kontrollbiopsien das Krankheitsbild der Krankheitsphase zugerechnet, der der Knochenmarksprozess am nächsten steht. Die Bewertung des erythropoetischen Zellgehalts im histologischen Knochenmarkpräparat bzw. im Sternalmarkausstrich erfolgte grundsätzlich immer vom gleichen Untersucher nach semiquantitativen Gesichtspunkten. Eine ge-

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Vergleichende Untersuchungen zur Erythrozytenkinetik und zur Knochenmarksmorphologie bei Patienten mit Osteomyelosklerose und Osteomyelofibrose

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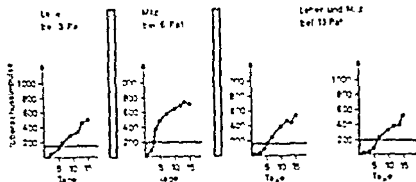


Abb. 1. Ort des maximalen Erythrozytenabbaus bei Patienten mit Myelofibrose und Osteomyelosklerose

kleinen Zahl von untersuchten Kranken keine bindenden Aussagen gestatten.

Material und Methoden

Bei 34 Patienten mit Osteomyelosklerose bzw. Myelofibrose erfolgte die Bestimmung der [illegible] Erythrozyten-Lebensdauer nach den Angaben von [illegible] und [illegible] [19, 5] [illegible] Messung von Oberflächenaktivitäten und Berechnung [illegible] nach dem Verfahren von Hughes-Jones und Szur [15]. Bei 16 Kranken wurden Untersuchungen zur Eisenkinetik nach den Angaben von [illegible] et al. [14] bzw. [illegible] und [illegible] [?] [illegible] Oberflächenaktivitätsmessungen über Leber, Milz, Herz und Kreuzbein durchgeführt.

Die Diagnose einer Osteomyelosklerose bzw. Myelofibrose wurde [illegible] durch die histologische Untersuchung eines [illegible] Knochenbiopsie [1] gewonnenen Knochenzylinders gesichert. Die Krankheitsstadien der Osteomyelosklerose wurden nach den Angaben von [illegible] [23] in 3 Phasen differenziert: 1. Die fibrös[illegible] Phase oder Stadium I, 2. die [illegible] Zwischenphase oder Stadium II und 3. die osteoplastische Stabilisierungsphase oder Stadium III.

In Übereinstimmung mit anderen Autoren [1, 11, 20, 27] wurde auf Grund langjähriger Verlaufskontrollen die Myelofibrose ohne Mitbeteiligung des Knochens vom Krankheitsbild der Osteomyelosklerose abgegrenzt. Auf die Problematik eines derartigen aus formalpathogenetischer Sicht bedingten Vorgehens kann in diesem Zusammenhang nicht eingegangen werden.

Wir sind uns bewusst, dass gelegentliche Phasen zwischen den einzelnen Krankheitsstadien erfasst werden, die nur bedingt eine Zuordnung in die angegebene Stadieneinteilung gestatten. Wegen der für die Vergleichsdarstellungen besseren Übersicht wurde nach mehreren Kontrollbiopsien das Krankheitsbild der Krankheitsphase zugerechnet, der der Knochenmarksbefund [illegible]
im histologis[illegible]
lich immer [illegible]

Tabelle I Erythrozytenkinetik bei Patienten mit Osteomyelosklerose und Myelofibrose

Parameter der Erythrozytenkinetik	↓	=	↑	insgesamt
Plasmaeisenclearance	3	1	12	16
Plasmaeisenturnover	1	4	11	16
Eiseninkorporationsrate	11	5	–	16
«Scheinbare» halbe Überlebenszeit	28	6	–	34

↓ = herabgesetzt, = = normal, ↑ = gesteigert

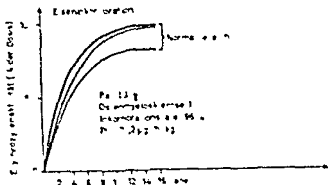
Tabelle II Lokalisation und Umfang der Erythropoese bei 16 Kranken mit Osteomyelosklerose und Myelofibrose

Erythropoese in	«effektiv»	«ineffektiv»
Knochenmark	7	2
Milz	5	5
Leber	3	8

naue Auszählung der erythropoetischen Vorstufen täuscht nur eine Pseudoexaktheit vor [33], die den semiquantitativen Aussagen nicht überlegen ist. Die Untersuchungsergebnisse zur Erythrozytenkinetik wurden mit klinischen Parametern und den Knochenmarksbefunden verglichen und die zu treffenden Aussagen durch statistische Analysen geprüft (Korrelationsanalyse, χ^2-Test, m × k Tafel).

Resultate

Die Untersuchungsergebnisse der Erythrozytenkinetik entsprachen den Angaben anderer Autoren [2, 17, 24, 30] (Tab. I). Die Mehrzahl der mit ^{59}Fe untersuchten Kranken wies eine beschleunigte Eisen-Clearance, einen erhöhten Eisenumsatz und eine verminderte Eiseninkorporationsrate auf. Bei ¾ der untersuchten Kranken war die «scheinbare halbe Erythrozytenüberlebensdauer» der roten Blutkörperchen verkürzt.



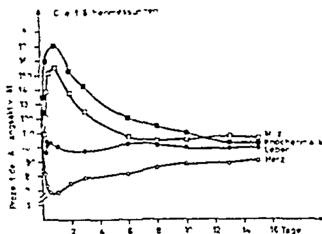


Abb. 7. Eisenkinetik (Inkorporation) und Oberflächenaktivitätsmessungen bei der Pat. I. J.

Die Oberflachenaktivitätsmessungen bei den mit Radioeisen untersuchten Patienten wiesen immer auf das Vorliegen einer extramedullären Erythropoese hin (Tab. II). Der Kurvenverlauf und die Berechnung bestimmter Indizes nach den Angaben von BRUNNER [2] liessen bei einzelnen Patienten mit überwiegend medullärer Blutbildung, bei der Hälfte der Kranken mit einer extramedullären lienalen Hämopoese sowie bei der Mehrzahl der Patienten mit hepatischen Blutbildungsstätten auf eine ineffektive Erythropoese schliessen. Drei Kranke zeigten eine verstärkte hepatische, 6 eine gesteigerte

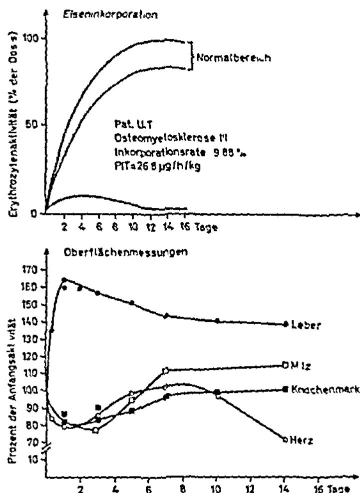


Abb 3. Eiseninkorporation und Oberflächenaktivitätsmessungen bei dem Pat K. T

lienale Erythrozytendestruktion, bei 13 Patienten fand sich sowohl in der Leber als auch in der Milz eine erhebliche Erythrozytoklasie, bei weiteren 6 Kranken muss ein gesteigerter Erythrozytenabbau generalisiert im RHS angenommen werden (Abb 1)

Die vergleichenden Darstellungen zwischen den Untersuchungsergebnissen der Erythrozytenkinetik und den morphologischen Knochenmarksbefunden sowie einzelnen klinischen Daten gestatten folgende Aussagen (Tab III)

1 Bei Patienten mit Osteomyelosklerose und Myelofibrose besteht zwischen der Eiseninkorporationsrate und dem Hämoglobingehalt

Tabelle III Verhalten der Parameter der Eisenkinetik im Vergleich zum Knochenmarksbefund und zu den Werten des roten Blutbildes

Verglichene Parameter		r	χ^2	f	p
Krankheitsstadium	– Clearance		3,99	4	>5%
	– Plasmaeisenumsatz		3,50	4	>5%
	– Eiseneinbaurate		6,[illegible]	6	>5%
Eiseninkorporationsrate	Hb	+0,573		14	<5%
	Erythrozytenzahl	+0,541		14	<5%
	Erythropoese (Hist.)	+0,575		14	<5%
	Erythropoese (Sternalmark)	+0,433		14	>5%
	Hämopoese (Hist.)	+0,673		14	<5%
Erythrozyteneisenumsatz	Erythropoese (Hist.)	+0,211		13	>5%
	– Hämopoese (Hist.)	+0,5[illegible]		13	<5%

sowie der Erythrozytenzahl zwar ein statistisch gesicherter, aber nicht sehr ausgeprägter Zusammenhang. Diese Aussage steht mit den Angaben von SZUR und SMITH [31] in Einklang, aber im Gegensatz zur Mitteilung von KESSE-ELIAS *et al.* [17].

2. Es lassen sich keine statistisch gesicherten Beziehungen zwischen den Krankheitsphasen und dem Verhalten der Eisenclearance, des Plasmaeisenturnover sowie der Eiseneinbaurate erkennen. Die Effektivität und das Ausmass der medullären, aber auch der extramedullären Erythropoese erscheint so vom Krankheitsstadium weitgehend unabhängig. Dieses Ergebnis einer funktionellen Betrachtung des erythropoetischen Systems stimmt mit den pathologisch-anatomischen Vergleichsbefunden der Arbeitsgruppe um PITCOCK [28] überein.

3. Es lässt sich eine positive Verbundenheit zwischen dem Verhalten der E[illegible]
der Eisenei[illegible]
Relation E[illegible]
Vorstufen im Sternalmarkausstrich nicht getroffen werden. Eine noch engere Beziehung ergibt sich zwischen der in toto beurteilten Hämopoese des Knochenmarks und der Grösse der Eiseninkorporation. Diese Aussage wird durch die bei Markhyperplasien im histologischen Präparat nur unzureichende Differenzierung der einzelnen Markzellen verständlich, so dass auch dem erfahrenen Untersucher ein

Tabelle IV. Ort der Erythropoese im Vergleich zu klinischen Parametern

	χ^2	f	p
Knochenmarkserythropoese (Hist.) – erythropoetische Knochenmarksaktivität erfasst mit ^{59}Fe	10,76	4	<5%
Hepatische Erythropoese – Krankheitsstadium	1,96	2	>5%
Hepatische, effektive Erythropoese – Krankheitsstadium	1,18	2	>5%
Lienale Erythropoese – Krankheitsstadium	4,16	2	>5%
Knochenmarkserythropoese – Krankheitsstadium	2,50	2	>5%
Effektive Erythropoese im Knochenmark – Krankheitsstadium	2,25	2	>5%
Lienale Erythropoese – Milzgrösse	6,69	2	<5%
Hepatische Erythropoese – Milzgrösse	6,09	2	<5%

gewisser Anteil der erythropoetischen Stammzellen der Beobachtung entgehen [33].

4. Die Ergebnisse der Oberflächenaktivitätsmessungen über dem Knochenmark bestätigen den Zusammenhang zwischen dem morphologischen und funktionellen Verhalten der medullären Erythropoese. Der Kurvenverlauf der Oberflächenaktivitäten über dem Knochenmark weist dann auf eine medulläre Blutbildung hin, wenn diese auch im histologischen Schnitt erfasst wird (Tab. IV).

Zwei Beispiele lassen ein derartiges funktionelles und morphologisch übereinstimmendes Verhalten der Erythropoese erkennen. Bei der 1. Patientin mit dem Stadium I einer Osteomyelosklerose liessen sich eine regelrechte Eiseneinbaurate (Abb. 2) und die Zeichen der Blutbildung in Knochenmark und Milz erfassen. Bei dem anderen Patienten mit einer Osteomyelosklerose im Stadium III fanden sich nur eine sehr stark herabgesetzte Eiseneinbaurate (Abb. 3) und die Merkmale einer ineffektiven hepatischen Erythropoese.

5. Auch die Ergebnisse der Oberflächenaktivitätsmessungen (Tab. IV) lassen ebenso wie die Befunde des Eisenstoffwechsels enge Beziehungen zwischen dem Ausmass der medullären Erythropoese bzw. dem Vorliegen von extramedullären Blutbildungsstätten in Leber und Milz und der Krankheitsphase vermissen, ein *in vivo*-Befund, der den pathologisch-anatomischen Untersuchungsergebnissen früherer Autoren [11, 28] entspricht. Diese Aussage bezieht sich sowohl auf das Vorliegen einer effektiven als auch einer ineffektiven Erythropoese. Sie kann die von PRIBILLA [30] aufgestellte These zur Abhängigkeit der

Tabelle 1 Vergleich der Erythrozytenüberlebenszeitbestimmung mit klinischen Parametern bei Patienten mit Myelofibrose und Osteomyelosklerose

	r	χ^2	f	p
Erythrozytenüberlebensdauer – Knochenmarkserythropoese (Hkt.)	−0.621		37	0.1 %
Retikulozytenzahl – Knochenmarkserythropoese (Hkt.)	+0.095		37	> 5 %
Erythrozytenüberlebensdauer – Retikulozytenzahl	−0.503		37	1 %
Erythrozytenüberlebensdauer – Krankheitsstadium		1.44	4	> 5 %
Lienale Erythrozytendestruktion – Krankheitsstadium		3.27	3	> 5 %
Hepatische Erythrozytendestruktion Krankheitsstadium		0.10	3	> 5 %
Lienale Erythrozytendestruktion – Milzgrösse		1.33	3	> 5 %

extramedullären Blutbildung vom Krankheitsstadium nicht bestätigen.

6. Es besteht ein eindeutiger negativer Zusammenhang zwischen der Milzgrösse und dem Nachweis einer extramedullären lienalen Hämopoese. Bei Patienten mit Riesenmilzen wiesen die Ergebnisse der Oberflächenaktivitätsmessungen nur selten auf das Vorliegen extramedullärer Blutbildungsstätten in diesen Organen hin. Zur Interpretation dieser Aussage müssen die in derartigen *Milztumoren* häufig starke Fibrosierung sowie die immer wieder eintretenden Milzinfarkte mit Beeinträchtigung der Milzfunktion in Erwägung gezogen werden [9, 28, 32, 33]. Ausserdem konnte bei Kranken mit sehr grossen Milztumoren häufiger eine hepatische Hämopoese erfasst werden, als es bei Kranken mit einer nur gering *vergrösserten Milz* der Fall war. Ob bei derartigen Patienten eine stärkere Einbeziehung der Leber in das Krankheitsgeschehen stattfindet [11] *oder* ein Übergang der extramedullären Blutbildung von der Milz *auf die Leber* erfolgt, kann zur Zeit nur zur Diskussion gestellt werden.

7. Zwischen dem Ausmass der *morphologisch* erfassbaren medullären Erythropoese und der Erythrozytenüberlebensdauer besteht eine

enge negative Korrelation (Tab V) Es könnte angenommen werden, dass durch die Steigerung der Erythropoese die verstärkte periphere Destruktion kompensiert wird Da sich aber zwischen der histologisch nachgewiesenen medullären Erythropoese und der Retikulozytenzahl kein Zusammenhang ermitteln liess, andererseits aber eine enge Beziehung zwischen dem Ausmass der Retikulozytose und der Erythrozytenuberlebensdauer besteht, muss angenommen werden, dass die medulläre Erythropoese dann weitgehend ineffektiv ist und eine gewisse Kompensation des gesteigerten Erythrozytenabbaus nur durch die extramedullaren Blutbildungsstätten erfolgt

8 Zwischen der Erythrozytenüberlebensdauer bzw dem Vorliegen einer gesteigerten Erythrozytendestruktion in Leber bzw Milz und dem Krankheitsstadium konnte keine statistisch zu sichernde Beziehung ermittelt werden

Trotz der nur mit einer gewissen Zuruckhaltung zu interpretieren den Aussagen statistischer Vergleichsuntersuchungen an biologischen Kollektiven lassen die vorliegenden Untersuchungsergebnisse erkennen, dass die histologischen und zytologischen Befunde des Knochenmarks sowie bestimmte klinische Parameter nur bedingt einen Rückschluss auf das funktionelle Verhalten der Erythropoese und der Erythrozyten gestatten Diese Folgerung wird durch die Komplexität der Anamiegenese bei Patienten mit Myelofibrose und Osteomyelosklerose verständlich, da sie aus der Kombination einer Erythrozytenminderproduktion oft verbunden mit einer ineffektiven Erythropoese [2, 10, 30] und einer gesteigerten lienalen oder hepatischen Erythrozytoklasie bzw eines beschleunigten Abbaus einer minderwertigen, kurzlebigen Erythrozytenpopulation [2, 12, 17, 24, 30] und einer verstarkten Blutzellspeicherung in der vergrosserten Milz [13, 31] resultiert Nur die kombinierte Anwendung morphologischer Untersuchungsverfahren und moderner Untersuchungen zur Erythrozyten kinetik konnen zum Verständnis der Anämie bei Osteomyelosklerose und Myelofibrose beitragen Es erscheint daher erforderlich, diese Diagnose grundsatzlich durch die histologische Untersuchung eines Knochenmarkschnittes zu stellen und gegebenenfalls den Krankheitsverlauf durch Kontrollbiopsien zu verfolgen, zusatzlich aber immer die nuklearmedizinischen Verfahren einzusetzen, um exakte Einblicke in das Wesen der schon fruhzeitig auftretenden Anämie zu gewinnen und so prognostische Aussagen zum Wert der einzuschlagenden therapeutischen Massnahmen zu treffen

Zusammenfassung

Bei 37 Patienten mit Myelofibrose bzw. Osteomyelosklerose wurde die Erythrozytenkinetik mit den histologischen und zytologischen Knochenmarksbefunden und einzelnen klinischen Daten verglichen. Eine direkte Abhängigkeit konnte zwischen den Parametern des Eisenstoffwechsels sowie der Erythrozytenüberlebenszeitbestimmung und den Krankheitsstadien nicht ermittelt werden. Dagegen besteht zwischen dem Vorliegen einer lienalen und hepatischen extramedullären Hämopoese und der Grösse der Milz ein statistisch gesicherter Zusammenhang. Die Aussagekraft derartiger Vergleichsuntersuchungen für das Verständnis der Anämiegenese bei Patienten mit Myelofibrose und Osteomyelosklerose wird betont.

Summary

The kinetics of erythrocyte movements were compared with bone marrow cytology and histology and certain other clinical data in 37 patients with myelofibrosis or osteomyelosclerosis. No direct correlation was found between iron metabolism and erythrocyte survival on the one hand and the different stages of the illness on the other. There is, however, a statistically significant relationship between the presence of splenic and hepatic extramedullary haemopoesis and the size of the spleen. The importance of such comparisons for understanding the origin of anaemia in patients suffering from myelofibrosis and osteomyelosclerosis is emphasized.

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The Ratio between Normal and Sensitive Erythrocytes in Heterozygous Glucose-6-Phosphate Dehydrogenase Deficient Women[1]

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The existence of two different populations of red blood cells in heterozygous glucose-6-phosphate dehydrogenase (G-6-PD) deficient women has been demonstrated [2, 6, 10]. Consistent with Lyon's hypothesis it is generally assumed that about half of the erythrocytes are normal and half are deficient. Nevertheless, accurate estimates of the ratio between normal and sensitive cells, drawn from a consistent number of appropriately chosen subjects, are not available and the possibility of a selective mechanism, modifying the theoretical ratio of one, cannot be ruled out.

A reliable method to discriminate between the two kinds of erythrocytes consists in treating blood *in vitro* with an oxydizing drug, such as menadione or primaquine at a concentration at which sensitive erythrocytes alone are damaged [10]. The injured cells are no longer viable and are rapidly sequestrated and destroyed if transfused into human beings. If the blood is previously labelled with ^{51}Cr the percent of residual radioactivity found after 24 hours equals the percent of non-enzyme-deficient cells [10]. We used this method over a long period of time, and we collected rather extensive material on this problem. These findings will be described in this paper.

Material and Method

Selection of subjects. Heterozygous subjects cannot be selected merely on the basis of enzymatic assays because G-6-PD levels in women are highly variable and a considerable

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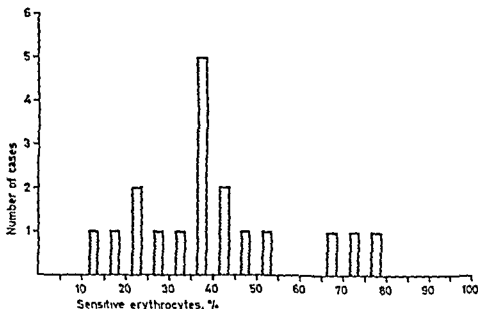


Fig 1 Percent of sensitive cells in the blood of 18 heterozygous G-6-PD deficient women

amount of heterozygous individuals may remain undetected Thus, we made our choice on a genetic basis and included in our sample (a) women who had both a normal and a G-6-PD deficient son, and (b) women who had a G-6-PD deficient father and a normal son or viceversa

Eighteen heterozygote women were studied for the detection of the percentage of sensitive cells This test was repeated in several different periods in 3 of the women, and was determined during the fava bean season in 8 of the women without clinical symptoms of haemolysis.

Determination of sensitive erythrocytes in G-6-PD deficient heterozygotes The blood was collected in sterile bottles with heparin and 2 mg of glucose/ml were added It was used fresh or stored for no longer than 12 hours at about 4°C. In all cases the blood was incubated for 3 hours at 37°C with menadione sodium bisulphite 1×10^{-4}M or with primaquine 8×10^{-4}M, labelled with ^{51}Cr (1 μC/kg of the recipient's body weight) and transfused into compatible volunteers Blood samples were taken 5 min and 24 hours later The percent of radioactivity of the second sample corresponds to the percent of non-deficient erythrocytes in the transfused blood. The accuracy, precision and reliability of the method have already been demonstrated [10]

Results and Discussion

On the basis of the collected material we tried to give an answer to the following three questions (1) Is the ratio between normal and G-6-PD deficient erythrocytes in heterozygous women determined at random? (2) Is the ratio constant in each subject, in basic conditions?

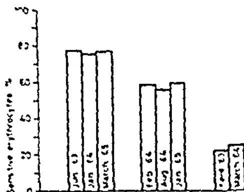


Fig 2 Percent of sensitive cells in the blood of 3 heterozygous women studied in different periods at intervals of several months

(3) Does the ingestion of fava beans regularly enhance a lysis of G-6-PD deficient erythrocytes?

We are not in a position to provide a definitive answer to the first question Figure 1 shows the distribution of the percent of sensitive cells in a group of 18 heterozygous women where any potential factor of haemolysis, such as fever, drug consumption, fava bean flowering and so on, are absent at that time The data are highly scattered The curve is skewed to the left the mean percent of sensitive cells is 40% ±17, instead of the expected 50% This may suggest a selective disadvantage for sensitive cells However, the difference between the actual and expected ratio is statistically unreliable

As to the second problem, we may assume that, in basic conditions, the ratio between sensitive and non-sensitive erythrocytes does not [illegible]

[illegible]

[illegible]

The third problem deserves a few preliminary comments It is currently assumed that severe fava induced haemolysis only occasionally occurs, even in G-6-PD deficient subjects who eat fava beans Someone still holds that fava induced haemolysis requires an allergic sensitisation, in addition to the enzymatic defect [3, 4, 5] PANIZON and VULLO [7] have already demonstrated that extracorpuscular factors, possibly related to the metabolism of fava-poison, are of

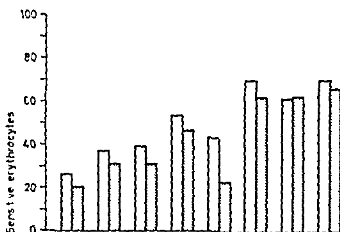


Fig. 3. Percent of sensitive cells in the blood of 8 heterozygous women studied outside (□) and during (■) fava season.

paramount importance in producing a severe haemolytic crisis. Nevertheless the effect of fava beans on G-6-PD deficient erythrocytes is not mediated [8]. Therefore it is possible that subclinical haemolysis is always present after ingestion of fava beans in G-6-PD deficient subjects [1, 9]. The study of the ratio between normal and sensitive erythrocytes in heterozygous subjects during the fava season is another possible approach to this problem. If this ratio decreases during this period, it can be assumed that a subclinical haemolysis, due to the ingestion or inhalation of fava beans – which are found everywhere in Sardinia – has occurred. Actually, a definite drop in sensitive erythrocytes occurred in 6 out of 8 cases studied both during and outside the fava season (fig. 3). Therefore the hypothesis that fava-induced subclinical haemolysis is the rule in sensitive subjects is confirmed.

Conclusions

The ratio between sensitive and normal erythrocytes is compatible with the hypothesis of a random inactivation of the X chromosome. This ratio is fixed in basic conditions. Environmental factors can temporarily modify this ratio without any clinical evidence; in particular, it seems that fava beans represent an important factor of subclinical haemolysis in almost all sensitive subjects.

Summary

In vivo survival curves of red cells damaged in vitro with menadione sodium bisulphite or primaquine are a reliable method for discriminating between normal and G-6-PD deficient erythrocytes. The ratio between the two kinds of erythrocytes was determined in 18 heterozygous G-6-PD deficient women. The study was repeated in 3 women in different periods and in 8 women during the fava season. The results indicate that the ratio between normal and sensitive erythrocytes is: (a) compatible with the hypothesis of a random inactivation of the X chromosome, (b) fixed in basic conditions, (c) modified in subjects who have come into contact with fava beans even when clinical haemolysis is absent.

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Behandlungsergebnisse mit Heparin und SP 54 bei autoimmunhämolytischen Anämien

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Bisher liegen zahlreiche Berichte über günstige Behandlungsergebnisse der hämolytischen Anämien mit Heparin vor. Erstmals behandelte im Jahre 1949 Owren [4] auf diese Weise einen Fall mit autoimmunhämolytischer Anämie, wobei die tägliche Verabreichung von 350 mg Heparin keine, eine wesentlich höhere Dosis jedoch eine günstige Beeinflussung der Hämolyse erkennen liess. Allerdings musste die Therapie infolge auftretender Blutungen vorzeitig abgebrochen werden. In der Folge wurde mehrmals von anderen Autoren über Erfolge mit der Heparintherapie berichtet, die erforderlichen Dosen waren jedoch ausserordentlich unterschiedlich und bewegten sich zwischen 50 und 800 mg pro Tag [1, 3, 5, 6, 7, 8, 10].

Keine Ergebnisse existieren bei der genannten Erkrankung mit dem Heparinoid SP 54 (Polyanion®, Bene Chemie), obwohl sich *in vitro* eine günstige Beeinflussung der Immunhämolyse feststellen lässt. So konnte die durch monophasische Kältehämolysine hervorgerufene Hämolyse bei Fall H. L. durch verschiedene Konzentrationen Heparin wie auch SP 54 dosisabhängig gehemmt werden, wobei die von beiden *Substanzen hierzu erforderlichen Mengen annähernd gleich waren* (Abb. 1). Gleichsinnige Resultate konnten wir auch mit dem Serum einer Patientin mit Wärmehämolysinen (Pat. H. E.) erzielen. Da die Beeinflussung des Blutgerinnungssystems mit SP 54 wesentlich geringer als mit Heparin ist, haben wir bei unseren Untersuchungen an Patienten hauptsächlich SP 54 angewendet.

Krankengut und Methodik

Die Untersuchungen wurden an 10 Fällen mit autoimmunhämolytischer Anämie vorgenommen, wobei 6mal Wärmeantikörper und 3mal Kälteantikörper nachgewiesen

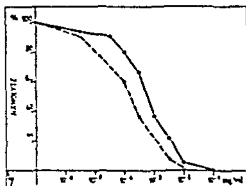


Abb. 1. Inkubation einer 50%igen Suspension gewaschener Erythrozyten der Blutgruppe O mit dem Serum der Patientin H. L. 30 min bei 4° C und anschließend 30 min bei Zimmertemperatur. Das Serum wurde vorher mit Salzsäure auf ein pH von 6,8 gebracht und mit verschiedenen Konzentrationen von Heparin und SP 54 vorbehandelt. ● Heparin ○ SP 54

werden konnten. Ein Fall nahm insofern eine Sonderstellung ein, als die zum Nachweis einer Immunhämolyse üblichen Teste negativ waren, die im Autohämolyseteat feststellbare Hämolyse jedoch durch Dekomplementierung und Zugabe von Heparin gehemmt werden konnte. Von den übrigen Fällen lag 3 mal die idiopathische und 6 mal die symptomatische Form der Erkrankung vor. Das SP 54 wurde bei 6 Patienten im Kurzversuch in einer Dosis von 400, 600 bzw. 700 mg im Laufe von 24 Stunden mittels einer Motorspritze infundiert. Sechs Patienten erhielten das SP 54 subkutan oder intramuskulär in einer Menge von 100 bis 400 mg durch 4–15 Tage täglich und 3 Patienten erhielten SP 54 peroral. Heparin (Heparin Novo®) wurde einmal mit einer Gesamtdosis von 180 mg über 24 Stunden infundiert, bei 3 Patienten in einer Höhe von 150–300 mg über einen Zeitraum von 4–28 Tagen subkutan in Depotform verabreicht (Tabelle I).

Patient E. C., männlich, 23 Jahre. 3 Monate vor Aufnahme erstmals Ikterus, seither zunehmende Anämie und mehrfach Transfusionen.

Befunde bei der Aufnahme: Ery 2,44 Mill., Hb 9,0 g%, Retikulozyten 420‰, Serumbilirubin 8,6 mg%, davon indirekt 7,1 mg%. Osmotische Erythrozytenresistenz: komplette Lyse bei 0,55%, inkomplette Lyse bei 0,85% NaCl. Direkter Coombs-Test 1:64 positiv. Kälteagglutinintiter 1:4. Leukozyten 6250, Diff. BB unauffällig. Sternalmark bis auf gesteigerte Erythropoese o. B. Scheinbare halbe Erythrozytenlebensdauer 4 Tage.

Diagnose: Idiopathische autoimmunhämolytische Anämie. Therapie: Depot-Heparin subkutan und nachher SP 54 peroral.

Patient H. F., weiblich, 34 Jahre. 4 Monate nach Entbindung eines normalen Kindes erstmals Müdigkeit, Ohrensausen, Schwindel. Bei der Aufnahme Subikterus, Blässe, unterer Milzpol 2 QF unter Rippenbogen.

Befunde: Ery 1,96 Mill., Hb 6,0 g%, Retikulozyten 214‰, Leukozyten 11000. Im Diff. BB vereinzelt Normoblasten. Im Sternalmark neben deutlicher Vermehrung der Erythropoese Zeichen einer megaloblastischen Reifungsstörung. Serumbilirubin 4,5 mg%, davon indirekt 3,1 mg%. Direkter Coombs-Test 1:64 positiv. Kälteagglutinintiter 1:32 positiv. Wärmehämolysine nachweisbar. Scheinbare halbe Erythrozytenlebensdauer

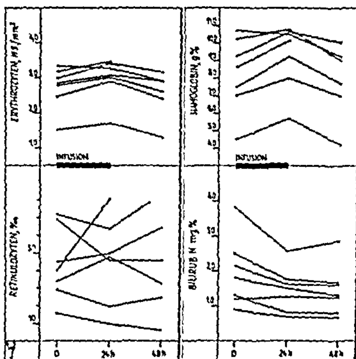


Abb. 2

3 Tage Auf Prednisolontherapie nur unzureichender Anstieg der Erythrozyten, daher 10 Monate nach Diagnosestellung Milzexstirpation, damit Teilerfolg erzielbar

Diagnose Idiopathische autoimmunhämolytische Anämie Therapie SP 54 zu verschiedenen Zeitpunkten als Infusion, intramuskulär und peroral

Patient B F, männlich, 55 Jahre seit 1 Jahr Bestehen einer chronischen Lymphadenose bekannt Wegen Subikterus und starker Anämisierung Spitalaufnahme

Befunde Erythrozyten 2,12 Mill, Hb 8,1 g%, Leukozyten 270 000, im Diff BB 93% kleine Lymphozyten Serumbilirubin 3,3 mg%, davon indirekt 2,7 mg% Direkter Coombs Test 1 256 positiv, Kälteagglutinintiter 1 4 Hepatosplenomegalie, zahlreiche harte, vergrösserte Lymphknoten

Diagnose Symptomatische autoimmunhämolytische Anämie bei chronischer Lymphadenose Therapie SP 54 intramuskulär

Patient F H, weiblich, 37 Jahre 2 Monate vor Aufnahme grippaler Infekt, danach zunehmende Blässe, Subikterus und Fieber Milzpol 5 QF unter dem Rippenbogen, Leber geringfügig vergrössert

Befunde Erythrozyten 1,98 Mill, Hb 4 8g%, Retikulozyten 106‰, Leukozyten 2400, im Diff BB zahlreiche Normoblasten, geringe Linksverschiebung der Granulopoese Im Sternalmark starke Vermehrung des erythropoetischen Anteiles Osmotische Erythrozytenresistenz komplette Hämolyse bis 0,45% Teilhämolyse bis 0 85 % NaCl Haptoglobine im Serum nicht nachweisbar Serumbilirubin 2,9 mg%, davon indirekt 2,0 mg% Indirekter Coombs-Test negativ, direkter Coombs-Test mit Antigammaglobulin, Antinongammaglobulin und mit Antigamma + Antinongammaglobulin negativ Hämolyse im Autohämolysetest durch Dekomplementierung und Heparinzusatz hemmbar Leberbiopsie Blutbildungsherde, kein Anhalt für Systemerkrankung

Name	Indikation	[illegible]	SP-54 Therapie: Mittl. Dosis (mg)	[illegible]	[illegible] Dosis (mg)	Dauer	[illegible]	Peroral Dosis (mg)	Dauer	Hepa Therapie: Mittl. Dosis (mg)	Depot Dosis (mg)	D	[illegible]
F. C.	Wärmeur[illegible] Hepatit[illegible]	4						1 0	10		1 1 0	4 7	• •
H. E.	Wärmeur[illegible] Hepat[illegible]	3	4 0	•	400 500 1 0	4 13 5	•	1 0 40	5 14				
I. M.	Wärme[illegible] Hepat[illegible]		30	•	4 0		•						
R. I.	Wärme[illegible] Symptom				30	15							
S. J.	Wärme[illegible] Symptom	4	4 0	•	4 0	4	•						
H. R.	Wärme[illegible] Symptom	"	0	•									
V. V.	Kä[illegible] Hepat[illegible]	1	0		4 0	9	•				31 50 31	4 5 5	• •
H. I.	Kä[illegible] Symptom	13						1	5		1 5		
F. V.	Kä[illegible] Symptom		0	•									
F. H.	[illegible]	4			0					5			

1 = Erf[illegible] Tag

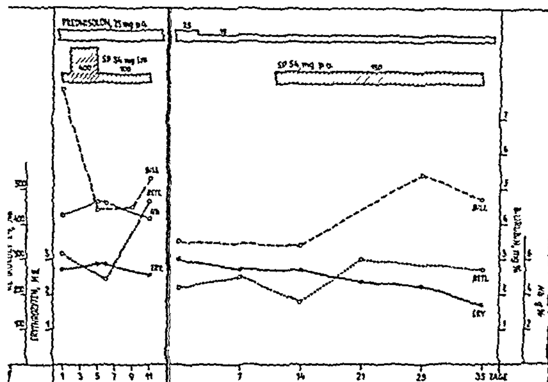


Abb 3 Patientin H E

Diagnose Immunhämolyse unklarer Genese Therapie Depot Heparin subkutan und später SP 54 peroral

Patient H L., weiblich, 69 Jahre erste Anämiesymptome 9 Monate vor der Klinikaufnahme, zahlreiche Transfusionen und Prednisolon Bei Aufnahme Subikterus, Blässe, mässige Lebervergrösserung und Konsistenzvermehrung, unterer Milzpol 2 QF unter dem Rippenbogen

Befunde Erythrozyten 1,7 Mill , Hb 6,1 g%, Retikulozyten 55‰, Leukozyten 3850, im Diff BB 66% nicht ganz typische Lymphozyten Serumbilirubin 4,0 mg%, davon 3,0 mg% indirekt Im Sternalmark Vermehrung lymphoider Retikulumzellen In der Leberbiopsie Infiltration der Periportalfelder mit lymphoiden Retikulumzellen, kleine herd- und streifenförmige Rundzellenansammlungen auch in den Sinusoiden Direkter Coombs-Test mit Antigammaglobulin negativ, mit Antinongammaglobulin I 16 schwach positiv, Kälteagglutinintiter gegen Zellen der Blutgruppe O über I 1024 positiv Nachweis monophasischer Kältehämolysine Scheinbare halbe Erythrozytenlebensdauer 13 Tage, die sedimentationsanalytische Untersuchung des Serums ergibt einen Anteil von 11% Globulinen mit einer Sedimentationskonstanten von 19 S

Diagnose Symptomatische autoimmunhämolytische Anämie durch Kälteantikörper bei Lymphoretikulose Therapie Heparin intravenös, später SP 54 subkutan

Patient H R., männlich, 70 Jahre Als Ursache einer seit 3 Jahren bestehenden hämolytischen Anämie wurde eine chronische Lymphadenose gefunden. Zahlreiche derbe Lymphknoten, mässige Leber- und Milzvergrösserung *Sternalmark, Leberbiopsie und* peripherer Blutausstrich ergaben für eine chronische Lymphadenose typische Befunde

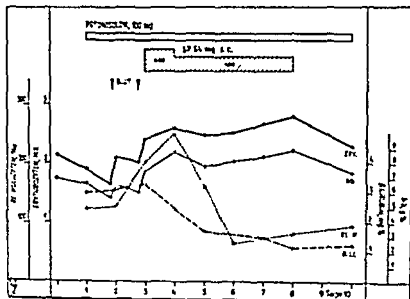


Abb. 4 Patient S. J.

Serumbilirubin 1,21 mg%, davon 1,0 mg% indirekt. Erythrocyten 2,7 Mill. Hb 10,0 g%. Retikulocyten 70‰. Leukocyten 6 8.0 Direkter Coombs-Test 1:64 positiv. Kälteagglutinintiter 1:8. Osmotische Erythrocytenresistenz normal. Scheinbare halbe Erythrocytenlebensdauer 7 Tage.

Diagnose: Symptomatische autoimmunhämolytische Anämie bei chronischer Lymphadenose. Therapie: SP 54 als Infusion.

Patient S. J., männlich, 67 Jahre. 4 Jahre vor Aufnahme Diagnosestellung einer chronischen Lymphadenose, in letzter Zeit zunehmende Anämie und Subikterus. Bei der Klinikaufnahme zahlreiche derbe bis kirschgroße Lymphknoten, unterer Leberrand 3 QF, unterer Milzpol handbreit unter dem Rippenbogen.

Befunde: Erythrocyten 2,3 Mill. Hb 6,5 g%. Retikulocyten 110‰. Leukocyten 67 300 mit 92% kleinen Lymphocyten im Diff. BB. Osmotische Erythrocytenresistenz: Teilhämolyse bei 0,7%, komplette Hämolyse bei 0,4% NaCl. Serumbilirubin 3,0 mg%, davon indirekt 2,4 mg%. Direkter Coombs-Test 1:512 positiv.

Diagnose: Symptomatische autoimmunhämolytische Anämie bei chronischer Lymphadenose. Therapie: SP 54 als Infusion und nachher subkutan.

Patient I. A., weiblich, 48 Jahre, vor 2 Jahren *Amputation mehrerer Finger* und vor einem Jahr Unterschenkelamputation wegen Gangrän, damals und auch jetzt schwere hämolytische Anämie.

[illegible]

positiv. Kälteagglutinintiter über 1:[illegible]

Diagnose Idiopathische autoimmunhämolytische Anämie durch Kälteantikörper. Therapie SP 54 als Infusion und nachher subkutan Depot Heparin zu einem anderen Zeitpunkt subkutan

Patient P A, weiblich, 37 Jahre 4 Monate vor Aufnahme zunehmend Blässe und Müdigkeit, fallweise Fieber, Subikterus Multiple Lymphknotenvergrösserungen Histo- [illegible]

[illegible]

0 65 mg% indirekt Direkter Coombs-Test 1 32 schwach positiv, Kälteagglutinintiter gegen Zellen der Blutgruppe O 1 512 positiv mit hoher Avidität

Diagnose Symptomatische autoimmunhämolytische Anämie durch Kälteantikörper bei Retikulosarkom Therapie SP 54 als Infusion

Patient T M, weiblich 79 Jahre Anämiesymptome erstmals 1 Jahr vor Aufnahme, mehrere Bluttransfusionen Auswärts Diagnosestellung einer autoimmunhämolytischen Anämie Auf Prednisolonbehandlung keine Besserung daher Transferierung Keine Lymphknotenvergrösserungen keine Milzvergrösserung

Befunde Erythrozyten 2 74 Mill, Hb 10 2 g%, Retikulozyten 144‰, Leukozyten 5400 im Diff BB Lymphozyten von 44% Im Sternalmark Vermehrung der Lymphozyten [illegible]

[illegible]

Infusion und später subkutan

Ergebnisse

Bei den 6 Patienten, die das SP 54 bzw das Heparin uber einen Zeitraum von 24 Stunden infundiert erhielten, wurden Erythrozytenzahl, Hämoglobin, Serumbilirubin, Retikulozyten und bei einigen Patienten auch die osmotische Erythrozytenresistenz vor, am Ende und 24 Stunden nach Ende der Infusion bestimmt Mit Ausnahme eines Falles mit Kälteagglutininkrankheit konnte jedes Mal ein Anstieg von Erythrozyten und Hamoglobin festgestellt werden, wobei der durchschnittliche Hamoglobinanstieg am Ende der Infusion 1,22 g% betrug Wenn man die bei diesen Erkrankungen vorhandene starke regenerative Tatigkeit des Knochenmarks in Betracht zieht und einen weitgehenden Stop der Hämolyse annimmt, dann korrelieren die auf diese Weise errechneten Daten gut mit den erhaltenen Ergebnissen Der auf das 6 8fache des Normalen gesteigerte Hämoglobinumsatz von 6–8 g/Tag ergibt einen Anstieg von annähernd 1 g% Hämoglobin pro Tag Einen Tag nach Absetzen der Medikation war bereits wieder eine deutliche Verminderung der Werte festzustellen In der Regel kam es unter der Infusion auch zu einem Bilirubinabfall, wahrend die Retikulozytenzahl einen unsicheren Erfolgsparameter

darstellte und keine einheitliche Veränderung erkennen liess (Abb. 2). Eine Veränderung der osmotischen Resistenz konnte nicht mit Sicherheit nachgewiesen werden.

6 Fälle erhielten SP 54 und 3 Fälle Heparin über einen Zeitraum von 3-28 Tagen subkutan bzw. intramuskulär verabreicht. Wurde das SP 54 in einer Dosis von 100 mg% (6stündlich 100 mg) gegeben, so war immer eine günstige Beeinflussung der Anämie festzustellen.

So erhielt die Patientin H. E. bei vorher kontinuierlicher Verschlechterung des Blutbildes durch 4 Tage das Präparat in der genannten Dosierung, wobei nun ein Anstieg der Erythrozyten von 2,65 auf 2,85 Mill., des Hämoglobins von 8,5 auf 9,2 g% und des Hämatokrits von 27 auf 30% festzustellen war. Bilirubin und Retikulozyten zeigten einen deutlichen Abfall. Die Verringerung der SP 54-Dosis auf 100 mg täglich hatte sofort eine gegenläufige Bewegung der verschiedenen Parameter zur Folge, und auch eine langdauernde orale Verabreichung von SP 54 in einer Höhe von 150 mg täglich konnte eine laufende Verschlechterung der Befunde nicht verhindern (Abb. 3). Auch die tägliche subkutane Injektion von 200 mg SP 54, welche die Patientin durch 10 Tage zu einem anderen Zeitpunkt erhielt, liess eine eindeutige Beeinflussung der Hämolyse nicht erkennen.

Der Patient S. J. wies eine symptomatische, durch Wärmeantikörper hervorgerufene, autoimmunhämolytische Anämie bei chronischer Lymphadenose auf. Nach 24stündiger Infusion von 600 mg SP 54 erhielt der Patient noch durch weitere 4 Tage täglich 400 mg SP 54 subkutan. In diesem Zeitraum stieg die Erythrozytenzahl von 2,4 auf 2,8 Mill., das Hämoglobin von 7,5 auf 9,2 g% an, während Bilirubin und Retikulozytenzahl abfielen. Auch hier kam es nach Absetzen der SP 54-Medikation wieder zu einer Verschlechterung, obwohl die Dosis der gleichzeitig verabfolgten Corticosteroide nicht verändert wurde (Abb. 4).

Bei der Patientin T. M. stieg während der 7tägigen subkutanen Verabreichung von 400 mg SP 54 täglich die Erythrozytenzahl von 2,7 auf 3,1 Mill. und das Hämoglobin von 9,0 auf 9,6 g% an, das Serumbilirubin fiel von 1,25 auf 1,0 mg% ab. Ebenso war bei der Patientin V. A., die an einer schweren progredienten Anämie bei chronischer Kälteagglutininkrankheit litt, unter der gleichen Dosierung von SP 54 durch 8 Tage ein geringer Anstieg von Erythrozytenzahl (2,8 auf 3,0 Mill.), Hämoglobin (8,8 auf 9,5 g%) und Hämatokrit (27 auf 30%) festzustellen. Zwei weitere Patienten, B. F. und F. H.,

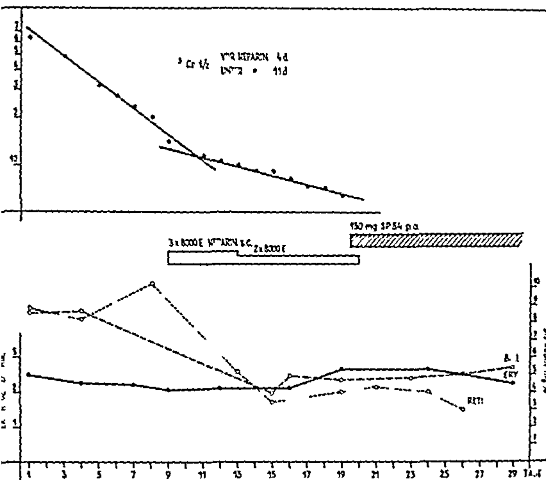


Abb. 5 Patient E. G.

erhielten SP 54 in einer Dosis von 200 mg täglich durch 15 bzw. 7 Tage subkutan. Es konnte in keinem dieser Fälle eine Besserung der Befunde festgestellt werden, obwohl ein bei einem dieser Patienten (F. H.) durchgeführter intravenöser Kurztest mit Heparin von Erfolg begleitet war. Die orale Medikation von SP 54, die bei 3 Fällen vorgenommen wurde, war unwirksam.

An Nebenwirkungen kam es bei der Injektion von 2 mal 200 mg SP 54 intragluteal bei Patientin H. E. zu Hämatomen an der Applikationsstelle, so dass in Hinkunft die Einzeldosen auf 100 mg beschränkt wurden und die subkutane Applikation der intramuskulären vorgezogen wurde. Bei Patientin P. A. trat am 4. Tag der Gabe von 1mal

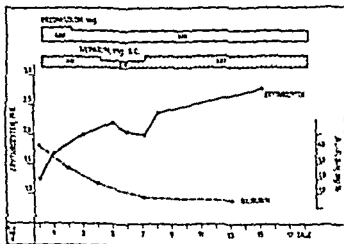


Abb. 6 Patientin V. A.

100 mg SP 54 subkutan eine oberflächliche Hautnekrose an der Applikationsstelle auf, so dass die Therapie unterbrochen werden musste. In den übrigen Fällen waren Nebenwirkungen der SP 54-Therapie nicht zu beobachten. Regelmässige Kontrollen der Prothrombinzeit, der Thrombinzeit und des Heparintoleranztestes ergaben nur geringfügige Beeinflussungen der Werte, so dass in keinem Fall die Therapie deswegen unterbrochen werden musste.

Heparin wurde in einem Fall (F. H.) im Laufe von 24 Stunden infundiert. Dabei stiegen die Erythrozyten von 3,0 auf 3,4 Mill., und das Hämoglobin von 8,6 auf 10,1 g% an, während das Bilirubin absiel. Die Dosis von 1000 E/h musste in diesem Fall nach 12 Stunden auf 750 E/h reduziert werden, da die Thrombinzeit bis über 60 sec angestiegen war. Drei weitere Fälle erhielten das Heparin in Depotform subkutan verabreicht.

Bei Patient E. G. mit einer schweren progredienten, im Coombs-Test positiven hämolytischen Anämie war unter einer Gabe von zunächst 220 mg und später 150 mg Heparin täglich keine weitere Verschlechterung, sondern eine mässige Besserung der Befunde festzu-

Heparintherapie wurde bei diesem Patienten ausserdem eine Bestimmung der Erythrozytenlebensdauer mit ^{51}Cr durchgeführt. Vor der Behandlung zeigte die Eliminationsgerade eine starke Neigung mit einer sich daraus ergebenden scheinbaren halben Erythrozytenlebensdauer von 4 Tagen. Mit Einsetzen der Heparintherapie änderte sich schlagartig die Neigung der Geraden, und die nun festgestellte Erythrozytenlebensdauer war mit 13 Tagen wesentlich länger (Abb. 5).

Die schwere progrediente Anämie bei Patientin V. A. wurde mit hohen Dosen Prednisolon behandelt. Gleichzeitig erhielt sie durch 4 Tage 280 mg Heparin subkutan verabreicht. Die Erythrozytenzahl stieg an, und das Serumbilirubin fiel ab. Durch weitere 4 Tage wurde nun das Heparin auf täglich 200 mg reduziert, worauf sich das Blutbild trotz Fortsetzung der Prednisolontherapie in gleicher Dosierung rasch verschlechterte und bei neuerlicher Steigerung der Heparindosis wieder besserte (Abb. 6). Es kann geschlossen werden, dass die Heparintherapie an der Besserung der Anämie einen wesentlichen Anteil hatte. Bei einer weiteren Patientin (H. L.) konnte eine eindeutige Beeinflussung der Hämolyse unter einer Dosis von 150 mg Heparin subkutan durch 7 Tage nicht festgestellt werden.

Diskussion

Bei Betrachtung der mit der genannten Therapie erzielten Ergebnisse können folgende Schlussfolgerungen gezogen werden:

1. Es lässt sich sowohl mit Heparin wie auch mit SP 54 eine günstige Beeinflussung der Hämolyse bei Fällen mit autoimmunhämolytischer Anämie erzielen, die allerdings nur während der Applikationsdauer anhält.

Es wurde berichtet, dass Heparin und Heparinoide bei Vorliegen eines Aldosteronismus zu einer *Diurese führen könnten* [2, 9]. Dagegen, dass bei unseren Fällen eine Bluteindickung zu einer scheinbaren Besserung der Blutbefunde geführt hat, spricht, dass in den meisten Fällen gleichzeitig auch eine Reduktion des indirekten Serumbilirubins festzustellen war und dass Fälle von hämolytischer Anämie anderer Genese eine Besserung der Befunde in jedem Fall vermissen liessen. Ein Anhaltspunkt für einen primären oder sekundären Aldosteronismus konnte bei keinem der behandelten Patienten gefunden werden

2. Die zur Beeinflussung der Hämolyse erforderlichen Dosen sind von Fall zu Fall verschieden. In der Literatur werden Heparinmengen

von 50–800 mg täglich genannt. Wir haben bei einem Patienten sowohl mit 220 wie auch mit 150 mg eine Besserung feststellen können, bei einem anderen Patienten erwies sich eine Dosis von 190 mg als unwirksam, während mit 260 und 320 mg ein deutlicher Erfolg zu erzielen war. Die Verabfolgung von SP 54 zeigte sich im intravenösen Kurztest mit 600 bzw. 700 mg mit Ausnahme eines Falles immer als wirksam, auch war die bei 3 Patienten vorgenommene Verabfolgung von 400 mg subkutan über einen längeren Zeitraum immer von einer Besserung der Befunde begleitet. Eine Dosis von 200 mg subkutan in 2 Fällen und 100 mg subkutan in einem Fall war unwirksam. Wie mit Heparin liess sich auch an einem Fall mit SP 54 eine deutliche Dosisabhängigkeit der Wirkung feststellen (H. L.). Die perorale Verabfolgung war immer unwirksam, selbst bei Dosissteigerung auf 500 mg täglich bei einer Patientin, die auf die intravenöse und subkutane Therapie gut angesprochen hatte. Wir vermuten, dass dies vorwiegend eine Frage der Resorption ist.

3. Das Heparinoid SP 54 bietet gegenüber dem Heparin insofern einen Vorteil, als es wesentlich weniger gerinnungswirksam ist, die zur Beeinflussung der Hämolyse erforderlichen Dosen jedoch nicht nennenswert höher liegen. Während in einem Fall die Infusion von stündlich 1000 E. (= 8,4 mg) Heparin eine so deutliche Beeinflussung der Gerinnungsbefunde zur Folge hatte, dass die Dosis nach 12 Stunden reduziert werden musste, waren mit der 4fachen Menge SP 54 (29 mg/h = 700 mg gesamt) während der 24stündigen Infusion lediglich geringfügige Veränderungen der Gerinnungsbefunde festzustellen, so dass in keinem der hochdosiert behandelten Fälle eine Reduktion der Dosis notwendig wurde. Es ist daher mit diesem Präparat eher die Möglichkeit gegeben, den zur Hemmung der Hämolyse erforderlichen Blutspiegel zu erreichen.

Zusammenfassung

An 10 Patienten mit autoimmunhämolytischer Anämie wurden Untersuchungen mit dem Heparinoid SP 54 und mit Heparin durchgeführt. Das SP 54 wurde als Infusion, subkutan oder peroral verabreicht. Heparin wurde entweder als Infusion oder subkutan in Depotform gegeben. Es zeigte sich, dass in genügend hoher Dosierung sowohl mit Heparin als auch mit SP 54 eine günstige Beeinflussung der Hämolyse erreicht werden kann, wobei die zur Hemmung der Hämolyse erforderlichen Dosen von SP 54 nicht wesentlich höher liegen als die von Heparin. Das SP 54 hat den Vorteil, dass die Beeinflussung des Blutgerinnungssystems wesentlich geringer ist und der erforderliche Wirkspiegel deshalb eher erreicht werden kann.

Summary

Investigations with heparinoid SP 54 and heparin were conducted in ten patients with auto-immune haemolytic anaemia. SP 54 was given as infusion, by subcutaneous injection or by mouth, and heparin by infusion or subcutaneous injection in depot form. It was shown that with sufficiently high dosage of either heparin or SP 54 a favourable effect on haemolysis could be obtained. The doses of SP 54 required to inhibit haemolysis were not appreciably higher than those of heparin. SP 54 has the advantage of a much less pronounced influence on the blood clotting system than heparin, so that it is easier to reach the effective level required.

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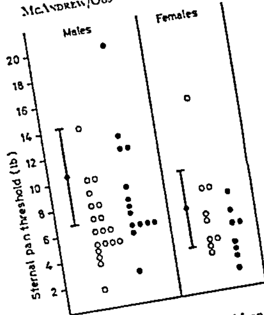


Fig 1 Sternal tenderness in patients with leukaemia I–●–I Mean ± SD of control patients, ○ Acute leukaemia, ● Chronic leukaemia

Table I Sternal tenderness in control patients

Age-group years	Sternal pain threshold (lb)							
	Males				Females			
	No	Range	Mean	SD	No	Range	Mean	SD
20–29	15	5–20	10.4	4.8	15	3–10	5.6	1.7
30–39	15	5–20	11.6	4.6	15	3–9	5.6	2.0
40–49	20	6–20	11.4	3.7	20	4–20	8.1	4.3
50–59	20	6–20	11.7	3.7	20	3–9	6.3	1.4
60–69	20	5–16	9.0	2.2	20	3–20	7.0	3.8
70–79	20	6–20	9.5	3.1	20	2.5–20	6.9	3.6
over 80	10	6–15	10.0	2.9	15	3.5–8	5.9	1.4
All ages	120	5–20	10.5	3.8	125	2.5–20	6.6	3.0

Results

The pressure required to produce sternal pain (sternal pain threshold) in the control patients, divided according to age and sex, is shown in table I. There was no trend towards a change in sternal tenderness

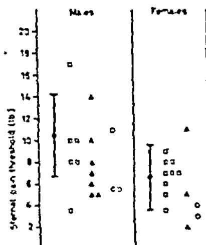


Fig 2 Sternal tenderness in patients with myelomatosis, pernicious anaemia and malignant infiltration of the marrow I ●–I Mean ± SD of control patients. □ Myelomatosis. ▲ Pernicious anaemia ○ Malignant infiltration of marrow

with age, but the men had significantly less sternal tenderness at each of the age-groups studied ($p<0.05$)

The mean sternal tenderness of both male and female patients with leukaemia was less than that of the control patients of the corresponding sex, but this difference reached the 5% significance level only in men The sternal tenderness of the individual patients with leukaemia, subdivided into acute and chronic forms, is compared with the mean of the control subjects for each sex in figure 1 Only 6 of the 50 patients with leukaemia had a sternal pain threshold below the range found in the control subjects

The sternal tenderness of patients with myelomatosis, malignant infiltration of the marrow and Addisonian pernicious anaemia is illustrated in figure 2 Only a single patient with myelomatosis and a single patient with pernicious anaemia had unusually low sternal pain threshold, the threshold was low in 4 of the 5 patients with malignant infiltration of the marrow

Comment

In clinical practice sternal tenderness is usually elicited by thumb or finger pressure In this study, using a more objective assessment of

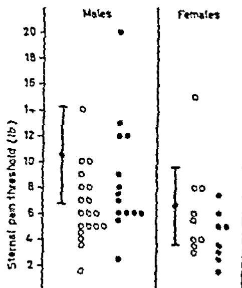


Fig 1 Sternal tenderness in patients with leukaemia I–●–I Mean ± SD of control patients, ○ Acute leukaemia, ● Chronic leukaemia

Table I Sternal tenderness in control patients

Age-group years	Sternal pain threshold (lb.) Males				Females			
	No.	Range	Mean	SD	No	Range	Mean	SD
20–29	15	5–20	10.4	4.8	15	3–10	5.6	1.7
30–39	15	5–20	11.6	4.6	15	3–9	5.6	2.0
40–49	20	6–20	11.4	3.7	20	4–20	8.1	4.3
50–59	20	6–20	11.7	3.7	20	3–9	6.3	1.4
60–69	20	5–16	9.0	2.2	20	3–20	7.0	3.8
70–79	20	6–20	9.5	3.1	20	2.5–20	6.9	3.6
over 80	10	6–15	10.0	2.9	15	3.5–8	5.9	1.4
All ages	120	5–20	10.5	3.8	125	2.5–20	6.6	3.0

Results

The pressure required to produce sternal pain (sternal pain threshold) in the control patients, divided according to age and sex, is shown in table I There was no trend towards a change in sternal tenderness

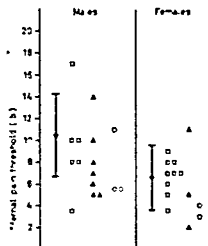


Fig 2 Sternal tenderness in patients with myelomatosis, pernicious anaemia and malignant infiltration of the marrow I ● I Mean ± SD of control patients, □ Myelomatosis, ▲ Pernicious anaemia ○ Malignant infiltration of marrow

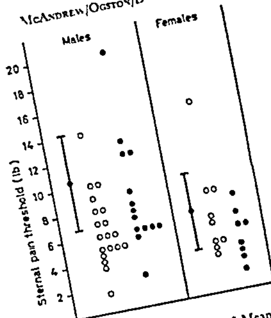
with age, but the men had significantly less sternal tenderness at each of the age groups studied ($p < 0.05$)

The mean sternal tenderness of both male and female patients with leukaemia was less than that of the control patients of the corresponding sex, but this difference reached the 5% significance level only in men. The sternal tenderness of the individual patients with leukaemia subdivided into acute and chronic forms is compared with the mean of the control subjects for each sex in figure 1. Only 6 of the 50 patients with leukaemia had a sternal pain threshold below the range found in the control subjects.

The sternal tenderness of patients with myelomatosis, malignant infiltration of the marrow and Addisonian pernicious anaemia is illustrated in figure 2. Only a single patient with myelomatosis and a single patient with pernicious anaemia had unusually low sternal pain threshold, the threshold was low in 1 of the 5 patients with malignant infiltration of the marrow.

Comment

In clinical practice sternal tenderness is usually elicited by thumb or finger pressure. In this study using a more objective assessment of



ernal tenderness in patients with leukaemia I-●-I Mean ± SD of control patients, leukaemia ● Chronic leukaemia

Table I Sternal tenderness in control patients

group s	Sternal pain threshold (lb) Males No	Range	Mean	SD	Females No	Range	Mean	SD
-29	15	5–20	10 4	4 8	15	3 -10	5 6	1 7
-39	15	5–20	11 6	4 6	15	3 - 9	5 6	2 0
0–49	20	6–20	11 4	3 7	20	4 -20	8 1	4.3
50–59	20	6–20	11 7	3 7	20	3 - 9	6.3	1 4
60–69	20	5–16	9 0	2 2	20	3 -20	7 0	3 8
70–79	20	6–20	9.5	3 1	20	2.5–20	6 9	3 6
over 80	10	6–15	10 0	2 9	15	3 5– 8	5 9	1 4
All ages	120	5–20	10 5	3 8	125	2 5–20	6 6	3 0

Results

The pressure required to produce sternal pain (sternal pain threshold) in the control patients, divided according to age and sex, is shown in table I There was no trend towards a change in sternal tenderness

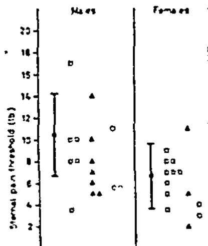


Fig 2 Sternal tenderness in patients with myelomatosis, pernicious anaemia and malignant infiltration of the marrow I●-I Mean ± SD of control patients. ○ Myelomatosis. ▲ Pernicious anaemia. Malignant infiltration of marrow

with age, but the men had significantly less sternal tenderness at each of the age-groups studied ($p < 0.05$)

The mean sternal tenderness of both male and female patients with leukaemia was less than that of the control patients of the corresponding sex, but this difference reached the 5% significance level only in men The sternal tenderness of the individual patients with leukaemia, subdivided into acute and chronic forms, is compared with the mean of the control subjects for each sex in figure 1 Only 6 of the 50 patients with leukaemia had a sternal pain threshold below the range found in the control subjects

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Comment

In clinical practice sternal tenderness is usually elicited by thumb or finger pressure In this study, using a more objective assessment of

sternal tenderness, we found that few patients with leukaemia had a degree of sternal tenderness outside the range found in control patients, and it is unlikely that the small increase in sternal tenderness found in a few leukaemic patients could be detected by the usual clinical method. We conclude that the physical sign of sternal tenderness is of very limited value in the clinical diagnosis of leukaemia. Sternal tenderness was also not notably increased in other conditions of increased marrow activity (megaloblastosis and myelomatosis). It may provide helpful evidence of metastatic malignant infiltration of the marrow, but the numbers in the present series are too small to reach a firm conclusion.

Summary

Sternal tenderness was measured in patients with leukaemia, myelomatosis, pernicious anaemia and malignant infiltration of the marrow, and compared with control subjects. Because of the wide scatter of results and the overlap of values with those of control subjects, it is concluded that sternal tenderness is not a useful clinical sign of leukaemia.

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A Screening Test for Red Blood Cell Catalase

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Acatalasia is a deficiency of catalase in the red blood cells and in various tissues, which was first described by Takahara [1]. The term hypocatalasemia is applied to intermediate values of catalase activity found in family members of patients with acatalasia. Acatalasia and hypocatalasemia are rare inherited conditions, for which a large Turkish population has been surveyed [2]. For this purpose a new, simple screening test suitable for field work was needed. This article describes such a screening test which is inexpensive, simple and which successfully detects acatalasic and hypocatalasemic individuals. It is particularly convenient in remote field survey, where no extensive biochemical facilities are available. Furthermore, it permits a large number of samples to be run within a short time, even by an inexperienced technician.

Method

Principle of the test. Whole blood is finally diluted 50 000 fold to test the catalase, while a sample for hemoglobin determination is obtained. The diluted blood sample is allowed to act on 7.5×10^{-4} M H_2O_2 buffer for 10 min at room temperature. The reaction is stopped by the addition of acid, and after neutralization the residual H_2O_2 is determined colorimetrically by a system of horse radish peroxidase-O-dianisidine. By comparing the color visually to standard colors corresponding to known enzyme units the blood catalase activity is assessed semiquantitatively. In normal individuals there is a linear relation between catalase activity and hemoglobin concentration [3, 4]. As the screening test simultaneously allows the determination of hemoglobin on the same blood sample used to test catalase, false hypocatalasemic values related to low hemoglobin are avoided, and only true cases of hypocatalasemia and acatalasemia can be detected.

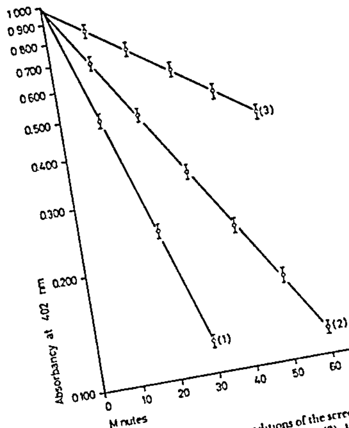


Fig 1 Time relation of catalase activity under the conditions of the screening test (1) Whole blood undiluted, (2) the same sample diluted ½ by saline, and (3) ¼ dilution ☿ denotes the average result of the triplicate determination on the same sample and ± SD

Material O Dianisidine, horse radish peroxidase (type 1), and bovine serum albumin (crystalline) were purchased from the Sigma Chemical Company, St Louis, USA All other reagents used were of CP or an equivalent grade, all compounds were dissolved in deionized water

Hemoglobin determination was carried out through the formation of cyanmethemoglobin [5], for which 20 μl of blood was withdrawn from the fingertip, and added to 5 ml of water prepipetted into a 130 × 14 mm test tube After, 50 μl of this hemolysate was taken for catalase assay at the end of the day, 2 drops of a concentrated hemolglobin reagent (10 g $NaHCO_3$, 500 mg KCN, 2 g $K_3Fe(CN)_6$ per 100 ml) were added and the tubes were shaken The results were visually compared to a series of standards ranging from 1 15 g% Hb prepared by the same method in the same size tubes from a sample with a known hemoglobin value, and covered with a cork Care was taken to select test tubes made of the same type of glass

The standards and samples prepared this way were stable for at least one week, and if further accuracy was desired they could be brought to the laboratory after a large group of samples had been collected and measured there at 540 nm. In cases where a hypocatala

Table 1 Absorption un [illegible] relation

Absorbance	Enzyme units per 100 ml blood[1]
1.000	0
0.900	200
0.805	400
0.725	600
0.610	900
0.520	1,200
0.425	1,600
0.335	2,000
0.245	2,600
0.145	3,300
0.069	4,300

[1] The enzyme unit was *defined in terms of velocity as follows* 1 *unit of enzyme* was equivalent to the activity that utilized 1 mMole of H_2O_2 per hour. The following formula was used $k_{cat} \ln(s_0/s) = V t$ where k_{cat} was a constant, s_0 initial H_2O_2 concentration (giving 1.000 absorbation under the conditions listed above) s the *concentration* of H_2O_2 at time t, and V the velocity

senic value was detected, repeated and conventional hemoglobin determination was carried out on a fresh blood sample taken from the subject

Enzyme assay The assay system should be freshly prepared on the morning of departure to the field. It consisted of 10^{-2} M potassium phosphate, 7.5×10^{-3} M H_2O_2 at pH 7 *Five ml* of this solution was pipetted into selected 150 × 16 mm pyrex tubes, all of the same colour. This solution is stable for 6 hours, and a stock solution of 7.5×10^{-3} M H_2O_2 can be kept cold for a week.

The screening test. A fresh blood sample for hemoglobin was obtained as described above. Immediately after the hemolysis had been completed 50 µl of this hemolysate was transferred to the tube containing the assay solution at room temperature (23–25° C). Catalase activity changes at temperatures around 23 C and false results are obtained at temperatures lower than 20° C and higher than 28° C. The chronometer was started, the tube was covered with a piece of parafilm and mixed by inverting. The micropipettes should be cleaned with *distilled water and acetone* between assays, and care should be taken to insure that both pipettes are clean and dry. After exactly 10 min of incubation at room temperature the reaction was stopped by the addition of 1.0 ml 2×10^{-1} M H_2SO_4 and mixing. The tubes may be kept covered and left at *room temperature* for 6 hours without significant loss of *residual* H_2O_2.

Color development [illegible] us *bovine serum albumin* kept at 0–4° C was diluted. O-dianisidine may be kept for one week in a dark bottle in the *refrigerator*, and darkened solutions should be discarded. Peroxidase will last for a fortnight if it is kept cold

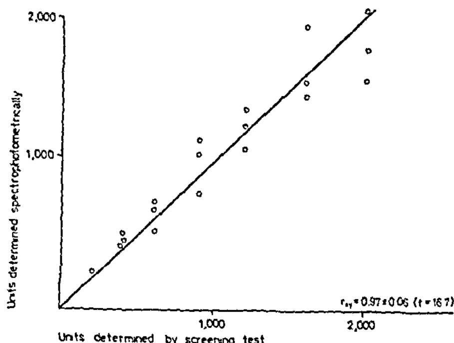


Fig. 2. Correlation between units of catalase activity determined spectrophotometrically and by the screening test. Standard correlation coefficients, standard deviation and the t value of the 20 samples are given

1.0 ml of the color solution was then pipetted into all the assay tubes and mixed by covering the tube with a piece of parafilm. Color development is very rapid, and should end within 2 min. At the beginning and end of the working day a blank should be prepared and run containing all the components of the assay system except the hemolysate. After the addition of acid and neutralization, color solution should be added to the blank, and color should be developed in the same way, after the full development of the color 0.5 ml 2 N HCl was pipetted into all the tubes and mixed.

Reading the results. Color was visually compared to standard tubes containing a known [illegible] a sample of O-dianis- [illegible] the same [illegible] an tubes at 402 nm. Dilutions were carried out in 1.2×10^{-3} N HCl to give the absorbencies corresponding to calculated enzyme units (table I). The standard color solutions were transferred to 150 × 16 mm test tubes and covered with a cork. These remain stable for at least a week, however a slight dark precipitate may form after a while in which case the tubes should be shakened and the precipitate dissolved before comparison. The blank assays should give a reading of about 1,000 absorbency, to ensure the validity of the enzyme assay; if this cannot [illegible] be expected. The assay mixtures may be [illegible] very stable at this stage, [illegible] ould satisfactorily detect patients with low blood catalase. When a case of low catalase was detected, the results were reconfirmed in a freshly drawn blood sample and sent to the laboratory

Results

The assay system, when run spectrophotometrically at 402 nm and plotted logarithmically was found to be linear for 60 min, which is to be expected, as the enzyme has a very large E_m value, and under the conditions described the disappearance of peroxide should follow first order kinetics This was confirmed in 20 normal blood samples, undiluted or diluted with fresh isotonic saline Ten minutes of incubation was found to be satisfactory for samples obtained from normal subjects and it was possible to detect hypocatalasemic subjects using this period of incubation [2] An example of the spectrophotometric assay is given in figure 1

The correlation between the units determined spectrophotometrically and the values obtained by the screening test was found to be satisfactory when tested for 20 normal subjects (fig 2) The accuracy of the screening test was determined in a double blind type of study by 3 different testers using various blood samples Individual variations in this type of study and also in repeated determinations with the same blood sample, were negligible with a standard deviation of $100 \pm 4\%$

The great dilution of the blood (final 50,000 fold) permits the specific assay of catalase without the interference of other activities due to the presence of undesirable substrates from hemolyzed cells (e g glutathione peroxidase) The catalatic activity of catalase has a large K_m [7] value, and under the conditions described here the disappearance or peroxide follows first order kinetics, velocity can also be calculated (table I) The rates were found to check theoretical calculations for 60 min at room temperature at the dilution selected The assays were run immediately after the collection of the blood sample, and for only 10 min hence no significant loss of activity could occur Furthermore, the activity was found to be stoichiometric to the enzyme concentration (fig 1) upon dilution

Comments

The screening test described has certain advantages over the usual assay system [6] The test is simple, inexpensive, needs no spec...

of 1,200 subjects could be successfully screened by 8 workers within 4 days [2]. It avoids elaborate testing by the conventional technique with a very large number of samples which requires good technicians in the field [6]. As true hypocatalasemic samples detected by this technique constitute only a few percent of the large population surveyed, only a few samples need to be brought to the laboratory to be checked accurately during a large scanning process, which is easy.

The test permits simultaneous hemoglobin determination in the field, and although it is only semiquantitative, a relevant value of catalase units per gram of hemoglobin may successfully be obtained; hence it is possible to avoid false results.

Summary

A suitable screening test for red blood cell catalase is described. The test was able to detect cases of hypocatalasemia successfully, and was easily applicable to remote field surveys for the presence of this rare inheritable disorder.

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Progress in Medical Genetics. Vol. VII. Editors: A. G. Steinberg and A. G. Bearn. Grune & Stratton, New York 1969. 351 p. Price: US $ 10.75.

The 7th volume of this valuable series upholds the high standards of its predecessors. The fare is varied. Four chapters provide critical reviews of the genetics of specific groups of diseases: R. B. McConnell on the gastrointestinal system, G. R. Fraser on thyroid disease, E. Eggermont on intestinal carbohydrate intolerance, and P. J. Fialkow on autoimmunity. F. H. Bach deals with histocompatibility in man, genetic and practical considerations. J. C. Dreyfus contributes a challenging theoretical discussion on the application of bacterial genetics to the study of human genetic abnormalities, which leads him to the conclusion that the existence of regulative genes has not been demonstrated in higher organisms and that the Jacob-Monod models of genic control in [illegible] do not fit the data on human genetic disease. Finally, D. H. Carr presents a survey of chromosomal abnormalities in clinical medicine which brings together in concise manner an immense array of findings in this much cultivated field. All articles are provided with extensive bibliographies and the volume ends with author and subject indexes.

Two suggestions may be directed to the editors. (1) References begin with the first author's name indented and the subsequent lines beginning without indentation. This interferes with easy recognition of the specific references. Why return to the method used in earlier volumes – and elsewhere – of starting with a non-indented first line and indented subsequent ones? (2) It would facilitate use and re-use of each chapter if it contained a table of contents. The extra space needed would seem to be worth the gain.

C. Stern, *Berkeley, Cal.*

Blutgerinnungsstörungen in der Gestation. Editor: H. Stamm. Bibliotheca Gynaecologica, Nr. 52. Karger, Basel/München/New York 1970. XI + 236 p., 67 Fig., 53 Tab., 2 Cpl. (Fr. 35.–, US $ 8.40, DM 35.–, [illegible]).

Störungen der Blutgerinnung und Hämostase vor, während und nach der Geburt umfassen thromboembolische Komplikationen, schwerste und dramatische Blutungen als Folge von Verbrauchskoagulopathien sowie eine Reihe von weiteren Störungen, die in Form von Vorträgen an der Jahresversammlung der Schweizerischen Gesellschaft für Gynäkologie vor 2 Jahren diskutiert wurden. Der Band enthält diese Übersichtsarbeiten, daneben auch zahlreiche Einzelbeobachtungen zu den genannten Themen. Die Mehrzahl der Vorträge interessieren primär den Kliniker. Insgesamt umfasst der Band das gesamte komplexe Spektrum der pathogenetisch teilweise noch sehr unklaren Gerinnungsstörungen während der Geburtsperiode.

F. A. Back, *Basel*

Immunology 1968. Proceedings of the International Conference on Immunology, Buffalo, N.Y., June 1968. N. R. Rose and F. Milgrom (ed.). Karger, Basel/München/New York 1969. XXXVIII + 371 pp., 14 fig., 97 tab. (Fr./DM [illegible], US $ [illegible]).

In June 1968, an International Conference on Immunology was held in Buffalo, to honour Prof. Ernest Witebsky and to inaugurate a new center for immunology under his direction. With this volume the 42 papers presented have been made generally accessible. As is fitting for the occasion, the papers are centered around some of the immunological problems the late Prof. Witebsky worked on, namely autoimmunity and tissue antigens.

The first group of 7 papers concerns 'Chemistry and Biology of Cellular Antigens'. Prof GRABAR gives a stimulating review of methods and new applications of the immunological approach to tissue antigens Two papers deal with endotoxins, in the first the group of WESTPHAL presents the latest data about the structure of the lipid A component The paper by PLESCIA et al, introducing an experimental model of myasthenia gravis, would be better placed in the later section on autoimmunity The second section of the book deals with 'Development and Control of the Immune Response'. GOOD and FINSTAD present, as usual, a lucid review about the relation between structure and function of the lymphoid system The physiological control of the immune response is dealt with in 2 papers by STERZL and THORBECKE. A review by FRANKLIN about proliferative disorders of plasma cells and lymphocytes is among the other papers. In the next section on 'Blood Group and Transplantation Antigens', RACE gives first a review about red cell specificity and human genetics, which is very useful for the immunologist who is not closely concerned with this field After 3 papers on the human HLA system, CUDKOWICZ enlarges the genetic spectrum by introducing the problem of hybrid resistance and parental specific transplantation antigens

From the section on 'Autoimmunity Models and Mechanisms' stimulating features emerge Homologous disease becomes fashionable once more and now serves to illustrate the relationship between autoimmune diseases and malignancies of the lymphoreticular system MELLORS takes the favourite natural model for autoimmune disease of recent years, NZB mice, and goes a step further by proposing viruses as the origin of both the autoimmune phenomena and the subsequent malignancies, with some new data. This view reconciliates many diverging facts in this and similar spontaneous models and has important implications for both genetic and aetiologic considerations in human diseases such as SLE. The next section about 'Tissue Specificity and Autoimmunity' is centered around renal diseases, giving in 3 papers a nice cross section, and around autoimmune aspermatogenesis The last section on 'Tumor Specific Antigens' is too short and incomplete to give even a glance of the variety in this large field

The proceedings of many meetings lack coherence This volume, however, is well organized and the main topics, tissue antigens and their implications for medicine and autoimmunity, are well covered without too much dispersion of topics. This book can be recommended to immunologists and clinicains equally

T L. VISCHER, *Basel*

Varia

1. Oesterreichische Haematologentagung

Wien, 23.-24 Oktober 1970

Thema Der Lymphozyt (Morphologie, Biologie, Funktion, Pathologie und Klinik der lymphatischen Reaktion

Kongresspräsident Prof Dr R. KLIMA, *Wien*.

Kongress-Sekretariat Doz. Dr H PIETSCHMANN, II Med Universitätsklinik, Garnisongasse 13, *A 1090 Wien* (Oesterreich)

Acta haemat. 43: 321-328 (1970)

Effects of Erythropoietin and Testosterone on Erythropoiesis in Bone Marrow of Isolated Perfused Hind Limbs of Dogs[1]

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Department of Pharmacology, Tulane University Medical School, New Orleans, La., and Department of Pharmacology, University of Tennessee Medical Units, Memphis, Tenn.

Testosterone has been known for several years to stimulate erythropoiesis in experimental animals [1, 2, 3]. However, the mechanism by which this agent stimulates erythropoiesis has been the subject of much recent controversy. Androgens have also been found to be useful in the treatment of clinical anemias which are refractory to other therapeutic agents [4, 5, 6]. It seems clear that at least one action of testosterone is to stimulate erythropoietin production [7, 8, 9]. We have found that testosterone stimulates production of erythropoietin (ESF) in isolated perfused dog kidneys [10]. On the other hand, direct effects of testosterone on bone marrow erythroid cells have also been reported [11, 12]. In addition, a synergistic action of testosterone and erythropoietin has been suggested [13, 14].

In order to clarify a possible direct effect of testosterone on erythropoiesis the effects of this hormone on bone marrow cells in isolated hind limbs of dogs perfused with blood containing testosterone and/or erythropoietin were studied.

Methods

All perfusions were performed in hind limbs from mongrel dogs weighing between 5.2 to 8.9 kg. The donor dogs used to obtain the blood which was added to the perfusion system ranged between 14 and 25 kg. All dogs used in the perfusion studies were previously heparinized with 1 mg/kg sodium heparin i.v. The perfusion system contained 500 ml of blood. All surgical procedures were carried out under pentobarbital anesthesia according to techniques which have been published previously [15–20].

[1] Supported by USPHS grant Am-07373, and USPHS International Post Doctoral Fellowship No. TW 1165 and * Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina.

Plasma samples were removed from the perfusion system initially and at the end of each experiment for erythropoietin assay All plasma samples were assayed for ESF in polycythemic mice according to the procedure of DeGowin et al [16] Each mouse was injected subcutaneously with 0 5 ml of plasma on the 4th and 5th day following removal from the low pressure tank, injected i v with 0.5 μc ^{59}Fe 2 days later and 48 hour ^{59}Fe incorporation in RBC determined

All hind limbs were perfused for 5–6 hours When the perfusions were terminated the femurs were removed and bone marrow smears and cell suspension counts prepared the same as the contralateral limb according to the technique described by Rekers et al [17] Blood with high levels of ESF (0 85 units/ml plasma) was obtained from dogs previously exposed to 18 hours of hypoxia (0 5 atmosphere) The same blood diluted approximately 4 times with blood from a normal donor dog was used in the perfusions with a low concentration (0 165 units/ml plasma) of ESF A water soluble form of testosterone sodium succinate[a] (0 2 and 0 1 mg/ml) was added to normal blood or used in combination with blood containing high or low levels of ESF

The isolated hind limbs were perfused with (1) normal blood (2) normal blood containing testosterone (0.2 mg/ml) (3) blood containing a high titer of ESF (0 85 unit/ml), (4) blood containing ESF (0 85 unit/ml) and testosterone (0.2 mg/ml), (5) blood containing a low titer of ESF (0 165 unit/ml), or (6) blood containing ESF (0 165 unit/ml) and testosterone (0 1 mg/ml)

Results

Figure 1 compares the percent change in total nucleated and total erythroid cells in all groups of isolated perfused hind limbs A slight decrease in total nucleated cell counts was usually seen in all groups of isolated marrow perfusions, except the group containing a low dose of ESF combined with testosterone However, perfusion of limbs with blood containing the high dose of ESF resulted in a significant ($P < 0.05$) increase in erythroid cell counts Femoral marrows perfused with blood containing a low dose of ESF showed a moderate increase in total erythroid cells A significant increase in erythroid cell counts was seen with the combinations of the low dose of erythropoietin and testosterone, when compared with the low dose of ESF alone Perfusion of marrows with normal blood alone or with blood containing testosterone resulted in a slight to moderate decrease in total erythroid cells

Figure 2 shows the results of bone marrow differential analyses in each group of isolated perfused femoral marrows Results are expressed as the percent change in pronormoblasts, basophilic normoblasts, polychromatic normoblasts and orthochromatic normoblasts As demonstrated in figure 2, femoral marrows perfused with blood

[a] Kindly supplied by Dr John Babcock from Upjohn Laboratories Kalamazoo Mich

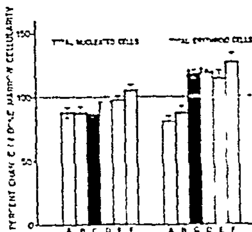


Fig 1 Mean total nucleated and total erythroid cell counts in femoral marrows of isolated dog hind limbs perfused with normal blood (A normal blood + testosterone (0.2 mg/ml) (B blood containing ESF (0.85 unit/ml (C) blood containing ESF (0.85 units/ml) + testosterone (0.2 mg/ml) (D blood containing ESF (0.165 units/ml (E) blood containing ESF (0.165 units/ml) + testosterone (0.1 mg/ml (F) The vertical lines at the top of each bar indicate standard error of the mean for each group (3–6 experiments The broken line represents the 100 percent base line values for the initial contralateral nonperfused limbs. *Significantly different from the initial control

containing the high dose of ESF (0 85 unit/ml), resulted in a significant ($P < 0.05$) increase in pronormoblasts and basophilic normoblasts and a moderate increase in polychromatic and orthochromatic normoblasts Perfusion of marrows with blood containing the low dose of ESF (0 165 unit/ml) also resulted in a significant ($P < 0.05$) increase in pronormoblasts, a marked increase in basophilic normoblasts, and a slight increase in polychromatic and orthochromatic normoblasts When testosterone was combined with the high dosages of ESF no significant increase in any marrow cell type was seen when compared with that of femoral marrows perfused with blood containing ESF alone However, when testosterone was combined with the low dosage of ESF a significant decrease in pronormoblasts and significant increases in basophilic and polychromatophilic normoblasts were seen when comparisons were made with the respective marrows perfused with the low dosage of ESF alone No significant difference was noted between erythroid cells in bone marrows from isolated

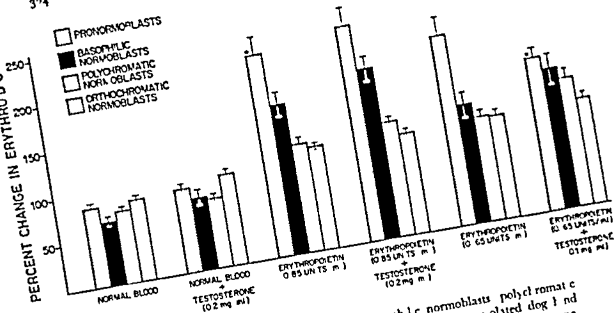


Fig 2. Mean percent changes in pronormoblasts, basophilic normoblasts, polychromatic normoblasts and orthochromatic normoblasts in bone marrows from isolated dog hind limbs perfused with blood alone or blood containing erythropoietin and testosterone. Standard errors of the mean are represented by the vertical lines at the top of each bar.
*Significantly different from the initial control.

limbs perfused with blood alone or blood containing testosterone. A slight to moderate decrease in all marrow cell types occurred in both groups.

Figure 3 shows the results of reticulocyte counts in blood perfusates of all isolated hind limb perfusion experiments. A marked increase in reticulocyte counts was observed in the blood perfusates from all groups of hind limb perfusions containing erythropoietin. The increases in reticulocytes were significant ($P < 0.05$) in all groups except the group perfused with the low dose of ESF. No change in reticulocyte counts was seen in the perfusate from isolated hind limbs perfused with normal blood alone or blood containing testosterone.

Mean perfusion pressure, femoral artery blood flow, hematocrits, arterial O_2 saturation, leucocyte counts and ESF concentration in plasma perfusates was monitored during all hind limb perfusions. A slight increase in the hematocrit was observed in all groups. Blood arterial oxygen saturation values were maintained near normal level throughout the perfusion. A moderate increase in arterial venous O

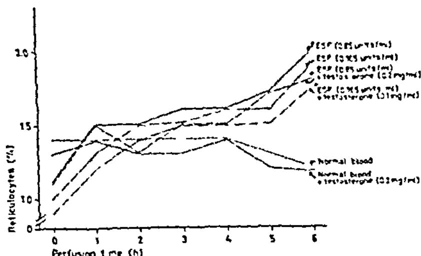


Fig. 3. Reticulocyte counts in perfusates of isolated hind limbs of dogs perfused with normal blood or blood containing erythropoietin and testosterone. *Significantly different from the initial control.

saturation difference was observed at the beginning as well as at the end of each experiment. No significant change was observed in WBC counts. A moderate decrease in ESF titers in plasma was usually seen at the end of the perfusion period in all experiments in which the blood perfusates contained an elevated level of ESF initially.

Discussion

The present findings indicate that testosterone alone does not exert a direct stimulatory effect on bone marrow erythroid cells in the isolated perfused dog hind limb. Isolated femoral marrows perfused with blood containing testosterone did not produce a significant effect on marrow erythroid cells, when compared with that of hind limbs perfused with blood alone. On the other hand, two different dosages of erythropoietin produced an increase in erythroid cells which was proportional to the dose of erythropoietin used in the perfusion system. These findings indicate that our isolated femoral marrow perfusion system is sufficiently sensitive to respond, as early as 5 hours perfusion,

to concentrations of ESF as low as 0 165 unit per ml of blood perfusate The moderate decrease in pronormoblasts seen during perfusion with the low dosage of ESF and testosterone may be due to an enhancement of maturation of this group of early erythroid cells The increases seen in the basophilic and polychromatic normoblasts may also be ... nergistic action of ESF and testosterone on the already ... ment This suggestion of an effect of the combination ... one on erythroid cells may be support for the reports of other workers [13, 14, 19] that testosterone increases the sensitivity of erythroid cells to ESF

We also found an increase in the rate of discharge of reticulocytes in perfusates from isolated femurs perfused with blood containing ESF These data confirm previous observations [20, 21, 22] that ESF, in addition to its action on stem cell differentiation, is capable of stimulating reticulocyte release ROH and FISHER [23] have also recently ... rabbits showed an early discharge of reticulocytes ... el of ESF during continuous exposure to hypox... ther support the hypothesis for a secondary action of erythropoietin on the reticulocyte compartment It has been estimated that the intermitotic generation time for each erythroid ... to 20 hours [24, 25] In view of these findings it ... into our blood perfusates after only 6 hou... e stem cell pool

It is also of interest to point out that there was a significant increase in total erythroid cells in isolated femoral marrows perfused with high doses of ESF The increase was most marked in the pronormoblasts and basophilic normoblasts Perfusion of femoral marrows with low doses of ESF also resulted in a significant increase in pronormoblasts These findings certainly support the concept that ESF stimulates stem cell differentiation However, an increase in the absolute numbers of polychromatic and orthochromatic normoblasts was also observed and is further evidence for a secondary action of erythropoietin on late erythroid cells

It seems clear from our previous studies that testosterone stimulates erythropoietin production in isolated perfused dog kidneys when the levels of renal erythropoietic factor were elevated by a previous exposure to hypoxia [10] Accordingly, we have postulated that the erythropoietic effects of testosterone are due, at least in part, to an increase

in kidney ESF elaboration. These results may also suggest that testosterone, in addition to its effect on stimulating kidney ESF production, may exert a synergistic action with ESF on marrow erythroid cells in vivo.

Acknowledgments. The authors gratefully acknowledge the technical assistance of Mrs. JUDY PARKER YOUNG and Miss JAYNE COX in these studies.

Summary

Testosterone alone does not exert a direct stimulatory action on bone marrow erythroid cells in the perfused hind limb of the dog. However, a slight to moderate increase in the effect of erythropoietin on erythroid cells was seen when testosterone was combined with erythropoietin in the perfusion system. The results of these studies suggest that the erythropoietic effects of testosterone alone are not due to a direct effect on erythroid cells but may be the result of a synergistic action with erythropoietin. Direct effects of testosterone alone and a more profound effect of the combination of ESF and testosterone on erythroid cells may possibly be demonstrated if the duration of perfusion could be extended over a more prolonged interval or a wider variety of dosages were employed. It is also difficult to extrapolate the present in vitro perfusion studies to the in vivo effects of testosterone reported in experimental animals and in the treatment of refractory clinical anemias which have responded to testosterone.

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Effect of Uracil and 2-Thiouracil on Leucocyte Production in the Rat

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The effect of 2-thiouracil (2TU) on leucocyte production is known mainly as a side effect of the therapeutic use of this compound and its methyl and propyl derivatives in the treatment of hyperthyroidism. Agranulocytosis is the most common reaction [1, 2]. It has been shown that 2TU can inhibit the mitosis of cultured human lymphocytes [3] and it may be that the fall in the white cell counts of some patients being treated with 2TU is due to its anti-mitotic effect.

The probable mode of action of 2TU is that it competes with uracil in the organism and thus blocks the pyrimidine pathways. It has been shown that in *Bacillus megatherium* the growth inhibition caused by 2TU can be reversed by the addition of equimolar amounts of uracil [4] and that the inhibition caused by 2TU in the fungus *Diplodia natalensis* can be overcome by treatment with uracil, thymine or cytosine [5]. 2TU also inhibits plant growth and this inhibition can be counteracted by uracil [6].

The experiments reported here were designed to investigate the effect of uracil and 2TU on leucocyte production in the rat.

Materials and Methods

An albino male rat was mated with a black hooded female. When the offspring weighed 50 g they were divided into 3 colonies which were allowed to inbreed. Colony 1 was given water containing 2 mg/ml of uracil, Colony 2, water containing 2 mg/ml of 2TU and colony 3 acted as a control. No restrictions were placed on the volume of liquid drunk by the rats and they were all allowed to eat the same pellet diet. Blood samples were obtained by cardiac puncture from 10 rats from each colony. Only rats which had been exposed to treatment from conception were used.

The blood for the white cell counts was prevented from coagulating by the addition of sequestrene, diluted with 1% acetic acid and the cells were counted in an improved Neubauer counting chamber. The blood films were stained with Jenner and Giemsa stains at pH 6.4 and 200 leucocytes per film were classified into granulocytes (i.e. neutrophils, eosinophils and basophils) and lymphocytes with monocytes.

Results

Table I shows the mean total white cell counts for each colony. Table II gives the mean differential counts for each colony together with the 95% confidence limits for the granulocytes calculated using the normal approximation to the binomial distribution [7]. Table III shows the absolute numbers of both groups of leucocytes calculated from the data of tables I and II.

Discussion

From table I it can be seen that while the mean counts for both the uracil and 2TU colonies are lower than the control colony the ranges given by the 95% limits show considerable overlap and there is, therefore, no evidence that either uracil or 2TU had any marked effect

Table I Total white cell counts per mm^3

Colony	Mean	Standard deviation	95% confidence limits
Control	8,875	1,761.9	5,421.7–12,328.3
Uracil	7,719	1,818.2	4,155.3–11,282.7
2TU	6,281	2,490.3	1,400.1–11,161.9

Table II Differential white cell counts

Colony	Differential mean		95% confidence limits of % granulocytes
	Lymphocytes and monocytes, %	Granulocytes %	
Control	88	12	10.6–13.4
Uracil	76	24	22.1–25.9
2TU	90	10	8.7–11.3

Table III White cell counts – absolute numbers per mm³

Colony	Lymphocytes and monocytes	Granulocytes
Control	7810.0	1050.0
Uracil	5066.4	18[illegible]6
2TU	5539.0	628.1

on the overall counts. From table II it can be seen that there is a slight reduction in the percentage of granulocytes in the 2TU colony when compared with that of the control. The chi-square calculated from a 2×2 table between the controls and the 2TU colony mean differential counts was 0.052 using Yates continuity correction. This is not significant. The difference between both the controls and the 2TU treated rats and the uracil colony tested in this way, however, gave chi-square results of 4.100 between control and uracil ($p < 0.05$) and 5.988 between 2TU and uracil ($p < 0.025$). From this it is possible to conclude that the addition of uracil to the drinking water of these rats caused a significant rise in the numbers of circulating granulocytes with a corresponding decrease in the number of lymphocytes and monocytes.

From table III it can be seen that there has been a fall in the absolute numbers of lymphocytes and monocytes with both uracil and 2TU, and that the absolute numbers of granulocytes has been increased with uracil treatment and decreased with 2TU treatment.

The decrease shown in the numbers of both groups of leucocytes in the rats treated with 2TU is consistent with the agranulocytosis and mitotic inhibition referred to previously. The decrease in the absolute number of lymphocytes and monocytes in the uracil treated rats suggests the possibility that excess uracil may have some inhibitory effect on lymphocyte production. The increase in the number of granulocytes present in the uracil treated rats may have a number of explanations. When uracil is supplied to the body in this manner it may reasonably be assumed that there is unlikely to be a deficiency of the pyrimidines, uracil, thymine or cytosine and that DNA or RNA production will not be a limiting factor in cell growth and replication. It is possible that the diet supplied to the rats was such that they were uracil-deficient although there is no direct evidence that this was so

and the control rats were certainly healthy. It should be noted, however, that in another experiment in which 5 young male albino rats were given the choice of drinking either water containing uracil or uracil-free water they chose to drink the water containing uracil.

It is possible that the effect observed was due to the oral route being used. This may have stimulated the gut flora and so indirectly induced a rise in the number of circulating granulocytes. Alternatively since it is generally agreed that the granulocytes are relatively short-lived it is possible that in conditions of pyrimidine excess they are more robust and able to survive longer in the body. It will certainly be the case that the precursor cells of the granulocytes will have a greater demand for DNA and RNA than the precursor cells of the much longer-lived lymphocytes and the change in differential count may be a reflection of the increased ability of the body to supply this demand when the animal is being fed excess uracil.

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Summary

Inbred colonies of rats were established from sibs in which one colony was treated with uracil in their drinking water and another with 2-thiouracil. A third colony acted as control. Both treated colonies had lower average white cell counts than the control and a significant rise in the number of granulocytes in the uracil treated rats was observed

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Heinzkörperbildung in Neugeborenenerythrozyten[1]

I. In-vitro-Studien über experimentelle Bedingungen und den Einfluss von Austauschtransfusionen

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Die Denaturierung von Hämoglobin in Erythrozyten durch verschiedene oxydierende Substanzen hat die Bildung von intraerythrozytären Innenkörpern zur Folge, die durch Supravitalfärbung in Form der bekannten Heinzkörper sichtbar gemacht werden können. Ähnliche Präzipitate lassen sich auch in stromafreien Hämolysaten erzeugen [26]. Es wird allgemein anerkannt, dass Heinzkörper aus denaturiertem Globin und Oxydationsprodukten des Hämanteils bestehen [31]. Während über die Denaturierungsvorgänge am Globinmolekül sowie über die Beziehungen zur Membran brauchbare Vorstellungen entwickelt wurden [1, 24, 26, 28, 44], ist der Ablauf in einzelnen Teilschritten mit Einbeziehung des Hämanteils noch weitgehend ungeklärt [2, 5, 17, 21].

Aus frühen klinischen Beobachtungen [16, 45] geht hervor, dass Erythrozyten von Neugeborenen und Frühgeborenen im Vergleich zu Erwachsenenerythrozyten eine ausgeprägte Neigung zur spontanen Heinzkörperbildung besitzen. Dieses Phänomen kommt besonders deutlich zum Ausdruck unter der Einwirkung von bestimmten Medikamenten (Sulfonamide, Vitamin K-Analoge, Naphtalin u. a.), deren Anwendung in der frühen Säuglingszeit das Krankheitsbild der toxischen hämolytischen Innenkörperanämie auslösen kann [4, 14, 16, 42] [illegible] erhöhte Heinzkörperempfindlichkeit von Neugeb[illegible] konnte *in vitro* erstmals von Künzer *et al.* [30] nachgewiesen u[illegible] anderen Autoren bestätigt werden [4, 7, 11, 20, 39, 40, 41, 43].

[1] Mit Unterstützung der Deutschen Forschungsgemeinschaft (Be 100/23, Kl

Ursache dafür ist nicht befriedigend geklärt. Bislang sind in experimentellen Untersuchungen darüber hauptsächlich die Erythrozytenenzyme [Übersicht bei 10, 19] und die Beziehungen zum fetalen (HbF) bzw. adulten (HbA) Hämoglobin berücksichtigt worden [11].

In der vorliegenden Arbeit wurde die Heinzkörperbildung durch Acetylphenylhydrazin unter verschiedenen *in-vitro*-Bedingungen vergleichend in Erythrozyten von Neugeborenen und Erwachsenen untersucht. Auf der Grundlage dieser Ergebnisse prüften wir Blutproben von Neugeborenen vor und nach Austauschtransfusion auf ihre Heinzkörperempfindlichkeit. Die zuletzt genannten Experimente sollten zur Klärung der Frage beitragen, ob Spendererythrozyten von Erwachsenen im Kreislauf von Neugeborenen gewisse Eigenschaften von Neugeborenenerythrozyten erwerben können. Aus den Ergebnissen lässt sich ableiten, dass die Heinzkörperbildung in Erwachsenen- und Nabelschnurerythrozyten prinzipiell gleich abläuft. Die Unterschiede sind quantitativer Natur. Weiterhin wird gezeigt, dass das Phänomen der verstärkten Innenkörperbildung nicht unbedingt ein Charakteristikum fetaler Erythrozyten[2] ist.

Material und Methoden

Sämtliche für die Untersuchungen verwendeten *Blutproben* wurden aus Vergleichsgründen zur gleichen Zeit venös abgenommen und mit 1/10 Volumen Natriumzitrat (3,8%) versetzt. Die Erythrozytenkonzentrationen zu vergleichender Proben wurden einander über die Hämatokritbestimmung durch Verdünnung mit dem jeweiligen Zitratplasma angeglichen. In den Versuchen im Zusammenhang mit den Austauschtransfusionen wurde ACD-Stabilisator verwendet.

Das β-Acetylphenylhydrazin (APH) wurde entsprechend dem Vorgehen von BEUTLER *et al.* [6] als 1% Lösung in isotonischem Kochsalz-Phosphat pH 7,4 verwendet und den Blutproben in einer Endkonzentration von 7,5 mM oder 15,0 mM zugesetzt. Die Inkubation erfolgte bei 37°C im Wasserbad.

Proben zur Herstellung von *Heinzkörperpräparaten* wurden in halb- bis einstündigen Intervallen entnommen und zur Färbung in der feuchten Kammer mit 0,5% Nilblausulfat in 0,9% Kochsalzlösung über 20 Minuten inkubiert. Die Zählung Heinzkörperhaltiger Erythrozyten wurde mit der Ölimmersion unter Zuhilfenahme eines quadratischen Zählfensters vorgenommen. Insgesamt wurden pro Präparat 1000 Zellen ausgezählt.

Bei den Untersuchungen über die *Abhängigkeit der Heinzkörperbildung von der Wasserstoffionenkonzentration* wurde die pH-Messung im Gesamtblut und in abzentrifugierten Erythrozyten im Astrup-Radiometer vorgenommen. Die Einstellung von Erythrozytensuspensionen auf verschiedene pH-Stufen (pH 8,3–8,4, 7,4 und 6,4) erfolgte durch wiederholtes Waschen mit entsprechenden isotonischen Phosphat-NaCl-Puffern. Die APH-Lösungen wurden für diese Versuche auf das gleiche pH eingestellt.

[2] Nomenklatur: Fetaler Erythrozyt = Erythrozyt des Neugeborenen unabhängig vom Gehalt an HbF (= HbF-Zelle) oder HbA (= HbA-Zelle).

Die *Abhängigkeit der Heinzkörperbildung von der Sauerstoffspannung* wurde in Schütteltonometern unter ständigem Durchfluss von definierten Gasgemischen (100% O_2, 96% O_2 + 4% CO_2, 92% O_2 + 8% CO_2, Luft = 20% O_2, 100% Stickstoff) geprüft. Blutproben von APH-Lösungen wurden 45 Minuten lang vor Versuchsbeginn mit dem entsprechenden Gasgemisch in getrennten Tonometern äquilibriert. Danach erfolgte die Zugabe der APH-Lösung zu der Blutprobe mittels Spritze durch einen Durchstechkorken.

Bei der überwiegenden Anzahl der Untersuchungen an *Blutproben* von Kindern mit Morbus haemolyticus neonatorum (MHN) *vor und nach Austauschtransfusion (AT)* diente neben dem jeweiligen Spenderblut in ACD-Lösung eine weitere Erwachsenenblutprobe als Kontrolle und Bezugswert für spätere Verlaufsuntersuchungen. Insgesamt konnten Blutproben von 11 Neugeborenen vor und bis zu 23 Tagen nach der Austauschtransfusion untersucht werden.

Als *Massstab* für die Bildungsgeschwindigkeit von Heinzkörpern wurde der Zeitpunkt gewählt, an dem 50% der Erythrozyten Heinzkörper enthalten (50% HK-Wert). Keine Berücksichtigung fand dagegen die Zahl der Heinzkörper pro Einzelerythrozyt. Als *Latenzzeit* wird jene Zeitspanne bezeichnet, die vergeht, bis in 5% der Erythrozyten Heinzkörper nach Beginn der APH-Inkubation auftreten (vgl. Abb. 1).

Ergebnisse[1]

1. Prinzipieller Ablauf der Heinzkörperbildung. Untersucht man die Heinzkörperbildung durch APH in Abhängigkeit von der Zeit unter den Bedingungen einer einfachen *in vitro*-Inkubation bei 37°C unter Luftatmosphäre ohne die Ansätze dauernd zu schütteln, dann ergibt sich folgender Ablauf (Abb. 1). Nach einer Latenzzeit erfolgt eine rasche Zunahme der Zahl der Erythrozyten mit Heinzkörpern. Etwa 30 Minuten nach Ende dieser Latenzzeit lassen sich im Durchschnitt in 50% der Erythrozyten (50% HK-Wert) Heinzkörper nachweisen, der 100% HK-Wert wird etwa innerhalb einer Stunde erreicht. Dieser am Ende der Latenzzeit beginnende Ablauf ist in Erwachsenen- und Nabelschnurerythrozyten prinzipiell gleich. Die Unterschiede in der Geschwindigkeit der Heinzkörperbildung sind allein durch die Dauer der Latenzzeit gegeben. Diese ist für Erwachsenenerythrozyten vierfach länger als für Nabelschnurerythrozyten. Die in Tabelle I dargestellten Daten wurden aus Ergebnissen von je drei Versuchen mit zwei verschiedenen APH-Konzentrationen errechnet. Für die 50% HK-Werte ergibt sich unter Einbeziehung der Latenzzeit eine etwa dreifach raschere Heinzkörperbildung in Nabelschnurerythrozyten, bei den 100% HK-Werten ist die Differenz gegenüber Erwachsenenerythrozyten mehr als doppelt so gross (Tabelle I).

[1] Die Ergebnisse wurden teilweise den Dissertationen von A.B. und M.K. entnommen.

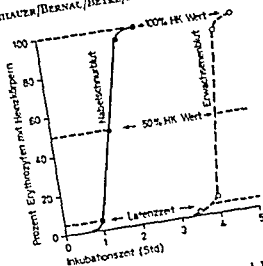


Abb 1 Ablauf der Heinzkörperbildung in Nabelschnur- und Erwachsenenerythrozyten Luftatmosphäre APH 7,5 mM in der Endkonzentration Latenzzeit, 50% und 100% HK-Werte sind eingezeichnet

Tabelle I Vergleich der Latenz und Bildungszeit von Heinzkörpern in Nabelschnur und Erwachsenenblut unter zwei verschiedenen APH Konzentrationen Die in Minuten angegebenen Werte stellen Mittelwerte aus je 3 Versuchen dar

	Nabelschnurblut		Erwachsenenblut	
APH Konzentration mM	7 5	15 0	7 5	15 0
Latenzzeit (min) 5% HK Wert	60	30	240	125
Bildungszeit (min) 50% HK Wert	90	50	255	150
Bildungszeit (min) 100% HK Wert	120	90	300	210

2 *Abhangigkeit der Heinzkorperbildung von der H-Ionenkonzentration* Die Ergebnisse der vergleichenden Untersuchungen an Nabelschnur- und Erwachsenenblut während der Dauer der APH-Inkubation sind in Form der Mittelwerte aus je 5 verschiedenen Ansätzen in Abbildung 2 dargestellt Während die Ausgangswerte in beiden Blutproben mit pH 7,22 und 7,23 noch dicht zusammen liegen, erfolgt in den ersten 30 Minuten nach Beginn der APH-Inkubation vor allem in den Ansätzen mit Erwachsenenblut eine deutliche pH-Zunahme Für das Nabelschnurblut liegen die pH-Werte über die ganze Versuchsdauer mit 0,1 pH-Einheiten konstant niedriger. Die Annäherung an die Werte des Erwachsenenblutes erfolgt 3½ Stunden nach Beginn der

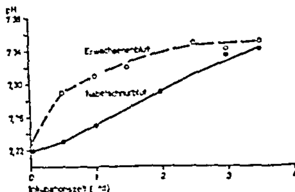


Abb. 2 Veränderungen und Differenzen der Wasserstoffionenkonzentration in Ansätzen aus Erwachsenen- und Nabelschnurblut während der Inkubation mit Acetylphenylhydrazin.

Inkubation Diese Entwicklung gilt nicht nur für das pH der gesamten Blutprobe, sondern auch für die Erythrozyten und das Plasma, deren pH Werte in diesen Ansätzen getrennt kontrolliert wurden

Werden Erythrozytensuspensionen durch wiederholtes Waschen mit isotonischem Phosphatpuffer auf differente Ausgangs pH Werte gebracht dann ist im Prinzip die gleiche Entwicklung zur Annäherung an den physiologischen pH Wert zu erkennen Nabelschnur- und Erwachsenenerythrozyten zeigen dabei keine prinzipiellen Unterschiede (Abb 3) Die quantitative Auswertung der Heinzkörperpräparate in diesen Versuchen ergab ausserdem eine grossere Geschwindigkeit der Heinzkörperbildung in höheren pH Bereichen Nabelschnur und Erwachsenenerythrozyten zeigten dabei ein gleichsinniges Verhalten in Form einer Verkürzung der Latenzzeit (Abb 4)

3 Einfluss der Sauerstoffspannung auf d e Heinzkorperbildung Verglichen wurde in dieser Versuchsserie die Heinzkörperbildung mittels APH in Erwachsenen und Nabelschnurerythrozyten in Gasgemischen, die Sauerstoff in einer Konzentration von 100 bis 0% enthielten Die APH Konzentration in den Ansätzen betrug 15 mM Aus den Kurvenverläufen in Abbildung 5 geht hervor, dass unter Abwesenheit von Sauerstoff (reine Stickstoffatmosphäre) sowohl in Erwachsenen- als auch in Nabelschnurerythrozyten über einen Zeitraum von 4½ Stunden keine Heinzkörperbildung erfolgt Höhere Sauerstoffkonzentrationen beschleunigen die Bildung von Heinzkörpern ganz erheblich

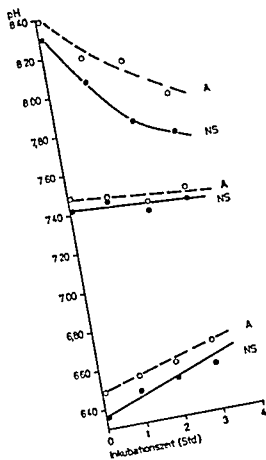


Abb 3 Veränderungen der Wasserstoffionenkonzentration in Ansätzen mit verschiedenem Ausgangs-pH während der APH Inkubation A = Erwachsenenblut NS = Nabelschnurblut

In einer Atmosphäre von 92% Sauerstoff mit 8% CO_2 sind noch Unterschiede in der Heinzkörperbildung zwischen Erwachsenen- und Nabelschnurblut nachzuweisen Bei 96% und 100% Sauerstoff verläuft der Prozess so rasch, dass Differenzen zwischen beiden Blutproben unter den gegebenen APH-Konzentrationen nicht mehr messbar sind Die Zunahme der Geschwindigkeit der Heinzkörperbildung erfolgt immer nur über eine Verkürzung der Latenzzeit Nabelschnur- und Erwachsenenerythrozyten verhalten sich dabei unter den verschiedenen Gasbedingungen prinzipiell gleich

4 Heinzkörperbildung in Erythrozyten von Neugeborenen vor und nach Austauschtransfusion Insgesamt wurden in dieser Serie die Blutproben von

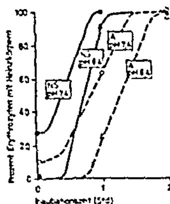


Abb. 4 Abhängigkeit der Geschwindigkeit der Heinzkörperbildung vom Ausgangs-pH. Gleichzeitig durchgeführte Untersuchungen bei pH 8,2 zeigten eine etwas raschere Heinzkörperbildung als bei pH 7,4. Diese Ergebnisse sind der Übersicht halber nicht eingezeichnet. A (gestrichelte Kurve) = Erwachsenenblut, NS (durchgezogene Kurve) = Nabelschnurblut.

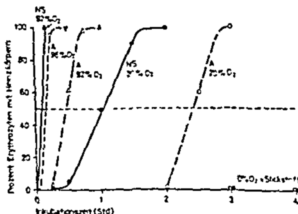


Abb. 5 Darstellung der Beziehungen zwischen Sauerstoffspannung (20–96% O_2) und Geschwindigkeit der Heinzkörperbildung in Erwachsenen (A) und Nabelschnurerythrozyten (NS). Die bei 3 und 4 Stunden Inkubation eingezeichneten Quadrate geben die *fehlende* Heinzkörperbildung in Stickstoffatmosphäre an.

11 Kindern mit Morbus haemolyticus neonatorum (MHN) infolge Rh-Inkompatibilität untersucht. APH wurde in einer Endkonzentration von 7,5 mM verwendet. Die Inkubationen erfolgten bei 37°C in

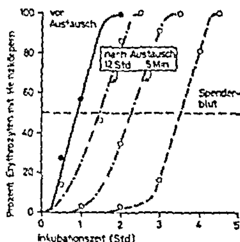


Abb. 6

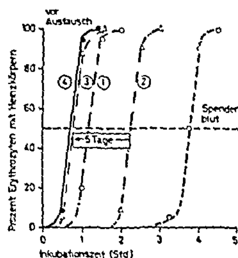


Abb. 7

Abb. 6 Veränderung der Geschwindigkeit der Heinzkörperbildung im Spenderblut verschiedene Zeiten nach Austauschtransfusion

Abb. 7 Verhalten der Heinzkörperbildung nach zweimaliger Austauschtransfusion (1) Lage der Kurve 12 Stunden nach erstem Austausch, (2) Heinzkörperbildung unmittelbar nach zweitem Austausch Fünf Tage später (3) entspricht die Heinzkörper-Bildungsgeschwindigkeit der in fetalen Erythrozyten (4) vor Austauschtransfusion

normaler Luftatmosphäre. Dabei konnten folgende Veränderungen im Ablauf der Heinzkörperbildung nachgewiesen werden (Abb. 6–8): Erythrozyten von Neugeborenen mit MHN vor Austausch zeigen keine Unterschiede in der Heinzkörperempfindlichkeit gegenüber Erythrozyten von normalen Neugeborenen. Desgleichen stimmen die Werte aus Spenderkonservenblut mit denen anderer Erwachsenenblutkontrollen überein. Nach der Austauschtransfusion ändert sich die Heinzkörperempfindlichkeit der transfundierten Erwachsenenerythrozyten derart, dass sie sich in zunehmendem Masse der von Neugeborenenerythrozyten angleicht (Abb. 6). Die erhöhte Empfindlichkeit zur Heinzkörperbildung ist bereits direkt im Anschluss an die Austauschtransfusion nachzuweisen und nimmt innerhalb der nächsten Stunden bzw. 3–4 Tage zu. Bei drei Kindern wurden Kontrollen bis zu 28 Tagen nach der Austauschtranfussion durchgeführt, wobei in keinem Fall wieder eine rückläufige Entwicklung in Form einer Angleichung an die Erwachsenenwerte nachzuweisen war. Auch nach zweimaliger Austauschtransfusion (Abb. 7) verhalten sich die trans-

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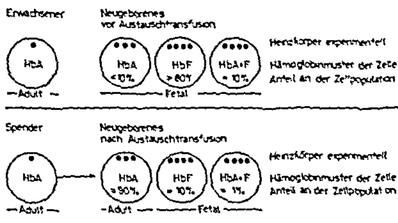


Abb 8 Schematische Darstellung der Heinzkörperbildung in Erythrozyten mit unterschiedlichem Hämoglobinmuster bei Neugeborenen vor und nach Austauschtransfusion. Die Zahl der in den Erythrozyten eingezeichneten Heinzkörpern soll das Mass für die Geschwindigkeit der Heinzkörperbildung darstellen. Die Bezeichnungen «Adult» und «Fetal» beziehen sich auf die Herkunft bzw. Bildungsorte der Erythrozyten

ten praktisch ausgeschlossen werden, da ein zweimaliger Blutaustausch den Anteil der fetalen Zellen auf etwa 5% vom Ausgangswert reduziert. Es ist demnach sehr wahrscheinlich, dass der transfundierte Erwachsenenerythrozyt in der neuen Umgebung andere Eigenschaften als vorher erwirbt. Dafür konnen theoretisch Veränderungen an allen Zellanteilen (Hämoglobin, Enzyme oder Membran) verantwortlich gemacht werden. Ein Wechsel im Hämoglobinmuster der Erythrozyten ist nicht denkbar. Dagegen sind Veranderungen von Enzymaktivitäten ebenso wie Änderungen in der chemischen Zusammensetzung der Zellmembran durchaus diskutable Moglichkeiten.

Unsere vorläufige Arbeitshypothese besteht darin, sekundär auftretende Veränderungen des Phospholipidgehaltes in der Membran der transfundierten Erythrozyten anzunehmen. Dies ist vorstellbar, weil einmal die Phospholipide zwischen Plasma und Erythrozytenmembran austauschbar sind [13, 34, 38]. Zum anderen erfolgt in der Membran wahrscheinlich keine *de novo*-Synthese von Phospholipiden [15, 32]. Darüber hinaus sind Unterschiede im Phospholipidgehalt des Serums und der Erythrozytenmembran von Neugeborenen im Vergleich zu Erwachsenen beschrieben worden, wobei die Angaben verschiedener Autoren allerdings erheblich variieren [3, 22, 37]. Inwieweit die nicht veränderte Lipidperoxydation von Neugeborenen-

erythrozyten unter der Wirkung von Wasserstoffperoxyd [8] gegen die vermutete Bedeutung der Erythrozytenmembran bei der erhöhten Heinzkörperempfindlichkeit spricht, bleibt weiteren Untersuchungen vorbehalten.

Zusammenfassung

Vergleichende in-vitro-Untersuchungen über die Heinzkörperbildung in Erwachsenen- und Neugeborenenerythrozyten durch Acetylphenylhydrazin zeigen eine unterschiedliche Länge der Latenzzeit, während der übrige zeitliche Ablauf in Nabelschnur- und Erwachsenenerythrozyten prinzipiell gleich ist. Nach Austauschtransfusion erwerben Erwachsenenerythrozyten im Kreislauf Neugeborener die Eigenschaft der erhöhten Heinzkörperempfindlichkeit in einem Ausmaß, wie sie für Neugeborenenerythrozyten charakteristisch ist. Die ursächlichen Zusammenhänge werden erneut diskutiert, und es wird auf die mögliche Bedeutung von Membraneigenschaften für die Heinzkörperempfindlichkeit hingewiesen.

Summary

This paper deals with in-vitro-Heinz body production in erythrocytes of adults and newborn infants by acetylphenylhydrazine. The mode of Heinz-body formation is principally equal in both age groups. The differences between the two types of red cells are due to different time intervals necessary for the beginning of Heinz body formation. The acquired susceptibility of adult donor cells in the circulation of newborn infants following exchange transfusion indicates the role of environmental factors. As a working hypothesis we suggest that the change in susceptibility is due to alterations in the phospholipid content of the red cell membrane

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Acta haemat 43 348-352 (1970)

The Activity of Glutathionperoxydase, Glucose-6-phosphate-dehydrogenase and Glutathionreductase in Sphaerocytes[1]

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The hereditary sphaerocytosis does not depend – as far as we know – on an intracellular enzyme defect, but on a primary defect of the membranes [4, 10] Since the reticulocytes in the blood increase during intravital haemolysis in that disease, this gives a variable system which may serve for an investigation of the activity of different enzymes during aging of the erythrocytes. Observations concerning the glucose-6-phosphate dehydrogenase (G 6-PDH), the glutathion reductase (GR) and other enzymes in blood rich in reticulocytes are published, but not concerning the glutathion-peroxydase (GSH-PX) Therefore, it seemed justified to investigate this.

Material and Methods

The activity of the G-6-PDH (1 1 1 49)[2] was measured using the enzyme kit produced by Biochemica Boehringer, Mannheim. The method follows the description by KORNBERG and HORECKER [5] The activity of the GR (1 6 4 2)[2] was determined according to BEUTLER and YEH [3], and the GSH PX (1 11 1.a)[2] according to PAGLIA and VALENTINE [7] All biochemical reagents were delivered by Boehringer Mannheim Perhydrol p a (H_2O_2) was purchased from E. Merck AG, Darmstadt. The enzyme determinations were performed under constant temperature of 25°C in cuvettes of 1 0 cm lightpath at 340 nm in a photometer 'Eppendorf' All enzyme activities are given in micromol substrat turnover in 1 min Each single value is based on a double determination

The erythrocytes were washed 3 times with saline 0.9% and the leucocytes were removed with the supernatant Haemolysis was achieved by adding an equal amount of distilled water to the concentrated erythrocytes followed by deep freezing and thawing The supernatant obtained after centrifugation was used for enzyme determinations after

[1] Supported by the 'Deutsche Forschungsgemeinschaft

[2] Enzyme commission list of enzymes 1965

diluting it to a haemoglobin content of 0.5 g%. The activity, obtained by this way for 1.0 g Hb, was converted to the activity for 10^{11} erythrocytes.

Determinations of enzyme activities were done on several occasions in 6 patients with hereditary sphaerocytosis, 5 patients (2 adults, 3 children) belong to 1 family

Patient E.T., fem., 8 years of age enzymes were studied during a haemolytic crisis before extirpation of the spleen as well as during reconvalescence At the time of the first examination the reticulocytes were elevated to 45‰, increasing further during the next days to 72‰. After extirpation of the spleen the reticulocytes decreased to 1 3‰.

Family M.-Sch. Both adults (=siblings as well as the 10 year old daughter of one of them had undergone splenectomy before because of haemolytic crisis. No haemolytic process was present when the blood was checked for enzymatic activity, the reticulocytes were not elevated (3–4‰) but the blood smear showed typical sphaerocytes. Two other children, aged 7 years (=cousins) were hospitalized for splenectomy because of recurrent haemolytic processes. Enzymes were studied before and after splenectomy. The reticulocytes were 25‰ and 29‰ respectively after the operation they came down to 1–5‰.

Results

An abnormal activity of enzymes was not found in sphaerocytosis as long as no haemolytic process was present During the time of an increased blood turnover (fall of the haemoglobin content, increased bilirubin and increased reticulocyte count) the activity of the G-6-PDH was increased on an average of 48%, of the GR of 35% and the activity of the GSH-PX on an average of 58% Table I shows the results in detail For comparison, the enzyme activities were measured in erythrocytes of 25 healthy children of the same age as the affected children The difference between the enzyme activity during active haemolysis and the enzyme activity at other times is highly significant for the GSH-PX ($P < 0.01$) and less significant for G-6-PDH ($P < 0.05$) and the GR ($P < 0.05$) Figure 1 demonstrates the course of the retic-

Table I Mean values and standard deviation of enzyme activities in erythrocytes (µMol substrate turnover / 10^{11} erythrocytes per minute)

	Healthy persons n=25	Patients with sphaerocytosis without increased reticulocyte counts n=8	with increased reticulocyte counts n=6
G-6-PDH	14 0±2.0	13.5	18 8
GR	10 6±2.3	9.8	13.2
GSH PX	200±30	239	380

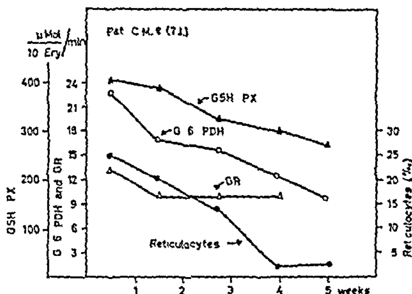


Fig. 1. Reticulocytes and erythrocytic enzymes during a haemolytic crisis in a child with hereditary sphaerocytosis

ulocyte count, and the enzyme activities in one patient. A certain parallelism between enzyme activity and reticulocyte count is present.

Following the procedure described by WITT *et al.* [11] fractions rich in reticulocytes and others poor in reticulocytes were obtained by centrifugation at 20,000×g: at the bottom the fraction poor in reticulocytes, and at the top the fraction rich in reticulocytes can be found. The two fractions were treated like the whole blood. In the top fraction (over 15‰ reticulocytes) the activities were higher than in the bottom fraction (less than 10‰ reticulocytes). The results are given in table II.

Discussion

The basic defect in hereditary sphaerocytosis concerns the membrane of the cells and manifests itself in an increased permeability for sodium [10]. No abnormal enzyme activities are known. Also the enzyme content of sphaerocytes does not differ from normal erythrocytes in our investigation. The increase of the G 6-PDH and the GR together with an increased haematopoiesis, documented by an elevation of the reticulocytes has been described [11]. An increased activi-

Table II Enzyme activities in fractions with high and low reticulocyte counts (μMol substrate turnover / 10^{3} erythrocytes per minute)

	Fractions with less than 10‰ reticul.	Fractions with more than 15‰ reticul.
G-6-PDH	13.9 ± 2.4	18.6 ± 4.3
GR	10.3 ± 3.1	17.9 ± 3.8
GSH PX	203 ± 21	330 ± 65

ty of erythrocytic enzymes points to a decreased mean age of erythrocytes, produced by a premature haemolysis of the old cells or by an augmented haematopoiesis [6]. Since the GSH PX is coupled to the redox system 'Glutathion' as close as the GR and secondarily to the GR also the G-6-PDH – it is not astonishing to find a similar behaviour of all three enzymes. In the investigations with fractions enriched in reticulocytes it was demonstrated, that a different content of enzymes in younger and older reticulocytes can be responsible for the differences found in the specimens of our patients. Preserving erythrocytes, one can find a slow decrease of the activity of different enzymes also *in vitro* [1, 7, 8].

The poor separation of the reticulocytes from the sphaerocytes received by us is remarkable. Usually it is possible to lower the number of reticulocytes to less than 2.5‰ in the bottom fraction [7]. But it is possible that under the chosen conditions no sufficient separation of reticulocytes and sphaerocytes can be achieved since both types of cells are more similar to each other in certain qualities i.e. the shape than reticulocytes and erythrocytes [9].

Summary

The activities of glutathionperoxydase, glucose-6-phosphatdehydrogenase and glutathionreductase show normal values in sphaerocytes. Elevated activities of all enzymes appeared combined with reticulocytosis during increased haematopoiesis. Reticulocytes contain a greater activity of all three enzymes than older erythrocytes.

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Acta haemat. 43: 353-359 (1970)

Homozygous Hemoglobin D Punjab

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Hemoglobin (Hb) D was first described in a patient with atypical hemolytic anemia who was a double heterozygote [1]. Biochemically Hb D is heterogenous and so far it has been found to occur in 4 forms: heterozygous Hb D trait, Hb S-D disease, Hb D thalassemia and homozygous Hb D disease. The last form is sufficiently rare to warrant this report.

Methods

Hemograms were obtained using standard techniques. Hb concentration was determined by the cyanmethemoglobin method. The microhematocrit was used for measuring packed cell volume. The modified methods of Simmons [2], Peters *et al.* [3] and Herbert *et al.* [4] were used to measure serum iron, total iron binding capacity and the folic acid level respectively. Erythrocyte reduced glutathione (GSH), glutathione stability, glucose-6-phosphate dehydrogenase (G-6-PD), and incubated erythrocyte osmotic resistance and autohemolysis were performed by the methods of Beutler *et al.* [5], Carrell and Prins as modified by Beutler [6], Zinkham [7] and Dacie *et al.* [8] successively. The Heinz body preparations were made as described by Dean *et al.* [9]. The starch gel electrophoresis was performed according to the method of Smithies [10] and the agar gel according to Robinson *et al.* [11]. Fetal hemoglobin value was determined by the alkali denaturation method of Singer *et al.* [12] and quantitative estimation of Hb A_2 was obtained by chromatography on diethyl amino ethyl cellulose (DEAE) [13]. The solubility of the reduced hemoglobin was determined at the 2.24 M phosphate concentration according to Jones and Huehns [14].

Case History

A.R.E., a 52-year-old male, was brought to our attention because his offspring had sickle cell HbD disease [15]. The propositus had no symptoms referable to possible hemolytic disease and the past history was unrevealing, particularly concerning pallor and icterus.

Table I Hematologic data of the patient

	Patient	Normal
Hb, g%	14 71	14–16
Hematocrit, %	43	42–46
Erythrocytes, 10^6/mm³	5 6	5–5.5
Mean cell volume, μm³	77	84 92
Mean cell hemoglobin, pg	26	27–32
Mean cell hemoglobin concentration, %	34 2	32 36
Reticulocytes, 10^3/mm³	63	50–100
Serum iron, μg%	122	80 120
Serum iron binding capacity, μg/100 ml	337	300 360
Folic acid, ng/ml	7 5	7 25
Bilirubin, mg%	0 8	less than 1 mg
Autohemolysis, % at 48 h		
without glucose	0 315	4
with glucose	0 187	0 8
G-6 PD, U/100 ml RBC	14	*110–160*
Glutathione, mg/100 ml RBC	23	78 8 ± 15 7
Glutathione stability	19	more than 40

Physical examination revealed a well developed male, weight 73.5 kg, and height 172 cm There was no jaundice or hepatosplenomegaly X rays of the long bones and skull were negative and the whole examination was within normal limits. Some of the laboratory findings are given in table I

The stained blood films revealed normochromic and normocytic erythrocytes with adequate platelets No target cells or nucleated red blood cells were seen The sickling test, made with 2% sodium metabisulphite and the direct and indirect antiglobulin (Coombs) tests were negative Heinz body and inclusion body content was not significantly different from the control cases Staining of the peripheral blood smear for the differential of hemoglobin S and D did not reveal any hemoglobin S [16] The bone marrow aspirate was hypercellular, and showed normal erythroid and myeloid maturation with the erythroid myeloid ratio of 1 3 5 Erythrocyte osmotic fragility was found to be slightly decreased at the beginning (fig 1) The solubility of the reduced hemoglobin of the patient was compared with those of patients with known sickle cell trait, sickle cell anemia and normal hemoglobin Although there was some precipitation with sickle cell trait and sickle cell hemoglobin, no precipitation was seen with the patient s hemoglobin as normal control Hb A_2 and fetal [illegible] original observation of electrophoretic mobility was confirmed and the location of the abnormality in the β-chain was identified

Chemical characterization of the hemoglobin was carried out by Drs Baudhall and Jones according to procedures described by Schneider *et al* [17] The abnormal peptide obtained from this hemoglobin by chromatography of the β-chain tryptic hydrolysate exhibits the unique behavior characteristic of Hb D Punjab (Los Angeles)

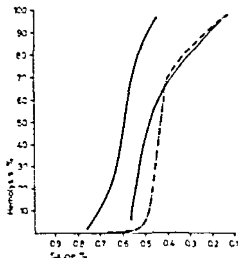


Fig 1 Incubated erythrocyte osmotic fragility of the patient was found t be slightly decreased in the beginning Normal range patient



Fig 2 Hemoglobin electrophoresis on starch gel at pH 8.6 (tris-citric acid). From top two sickle cell trait propositus control



Fig. 3. Agar gel electrophoresis at pH 6.45 (citrate-phosphate). *From top:* Control, Hb S-D disease with increased Hb F, sickle cell trait, propositus.

a) The abnormal tryptic peptide, β T-13, when chromatographed on a cation-exchange resin, is eluted more rapidly than the corresponding peptide from Hb A.

b) During chromatography in buffers in acid pH range, the abnormal peptide shows almost no color with ninhydrin, and the N-terminal residue is not acted upon by aminopeptidase M.

c) After hydrolysis with 6 N hydrochloric acid, the amino acid analysis of the abnormal peptide shows the same amino acid composition as normal β T-13.

This behavior can be explained by the presence of glutamine instead of glutamic acid at position 121 of the β-cahin which forms the N-terminal residue of peptide β T-13 after tryptic hydrolysis of the chain. In acid solution, the glutaminyl residue cyclizes and no longer reacts with certain enzymes or ninhydrin. Hydrolysis with hydrochloric acid converts it to glutamic acid.

Since the β T-1 peptide was normal, the presence of Hb S may be eliminated. Chain-separation gave only one β-chain peak. The α-chain was shown to be normal by hybridization of the hemoglobin with canine hemoglobin.

Family study indicated that the patient's wife had sickle cell trait and all four of his live offspring had sickle cell-hemoglobin D disease. Unfortunately, his parents and siblings were not alive and could not be investigated (fig. 4).

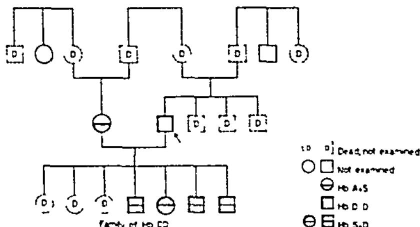


Fig 4 The family pedigree The arrow indicates the propositus)

Discussion

A hemoglobin which does not cause sickling, but which is indistinguishable from Hb S by conventional methods of electrophoresis and chromatography, is called Hb D However, this hemoglobin could be separated from Hb S on agar gel at 6 15 [11] The abnormal hemoglobin found in this patient was, by definition, hemoglobin D, shown to be Hb D Punjab (Los Angeles) ($\alpha_2\beta_2$ 121 Glu→Gln)

As has been mentioned, Hb D shown by electrophoresis and chromatography, is heterogenous Among others, hemoglobin Dα [18] and the two biochemically different hemoglobins Dβ, D Ibadan ($=\alpha_2\beta_2$ 87 Lysine) [19] and D Los Angeles ($\alpha_2\beta_2$ 121 Glu→Gln [20], are fairly well documented types

In general the patients with hemoglobin S-D and thalassemia-hemoglobin D disease, have moderate degree hemolytic anemia But the cases which are only heterozygote and homozygote for hemoglobin D do now show any specific abnormality related to this abnormal hemoglobin [17] There have been at least four reported cases of homozygous hemoglobin D disease in the literature [21–23] The two siblings reported by Stout *et al* [23] also had Hb D Los Angeles, but both of these patients had moderate degree anemia, high percentages of target cells and no appreciable amounts of A_2 in some members of the family The possibility of hemoglobin D α thalassemia

combination which could not be distinguished from homozygous Hb D disease by electrophoresis might be entertained.

It must be emphasized that an electrophoretic pattern of almost 100% Hb D does not alone prove homozygosity for this pigment. For this reason definite diagnosis of homozygous Hb D disease cannot be made without family studies in addition to electrophoretic pattern and Hb A_2 determination. Bird and Lehmann's case [21], who also had Hb D Los Angeles, is a good example for the importance of the family study in the diagnosis of homozygous Hb D disease. That sikh soldier's family became available to the authors many years later and the erroneous diagnosis of homozygous Hb D disease of this patient was corrected as hemoglobin Dβ-thalassemia [21].

In the case presented here, all the laboratory studies do not contradict homozygosity. In addition, all four living children had D genes which is more evidence that the propositus had pure hemoglobin D disease. However, it would be more convincing if his parents and/or siblings had been available for examination.

Although the association of homozygosity of Hb D with erythrocyte G-6-PD deficiency is unique, it seems to be purely coincidental. In spite of this combination the patient did not present any evidence of hemolytic anemia, but erythrocyte survival could not be performed.

All the previous four cases of homozygous hemoglobin D disease showed appreciable amounts of target cells. However, this more common finding was not detected either in the propositus or in the other family members with this hemoglobinopathy [15]. Erythrocyte osmotic fragility carried out in Chernoff's case [22] was significantly decreased, but it was not marked in our patient.

Acknowledgement. I am indebted to Drs. Brimhall and Jones of the University of Oregon Medical School for their kindness in analysing the patient's hemoglobin.

Summary

The case of a Turkish man with very probable homozygous hemoglobin D disease, whose offspring had hemoglobin S-D disease, is reported with laboratory detail. Investigations showed that he was hemoglobin D Punjab (Los Angeles) ($\alpha_2\beta_2$ 121 Glu→Gln). Although he also had erythrocyte glucose-6-phosphate-dehydrogenase deficiency, there was no present evidence and no past history of hemolysis.

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H. Mast: **Morbus haemolyticus neonatorum.** Additamentum ad vol. 170 Z. Geburtsh. Gynäk. Enke, Stuttgart 1969. VIII+96 S., 21 Abb., 22 Tab. DM 27.–

Die Behandlung der Blutgruppen-Inkompatibilität ist heute nur noch bei schwerer Sensibilisierung problematisch. Vor allem die schon in früher Schwangerschaftszeit einsetzende Hämolyse gefährdet den Fötus, der schon intrauterin lebensbedrohlich erkrankt oder abstirbt. Seit den bahnbrechenden Arbeiten des Neuseeländers Liley nehmen sich dieser schweren Fälle an den meisten Orten die Geburtshelfer an. Die vorliegende Broschüre berichtet über die Erfahrung der Universitäts-Frauenklinik Münster (Westfalen). Der Autor folgt den Richtlinien von Liley. Nach gründlicher Schilderung der Methoden und des operativen Vorgehens stellt er die Ergebnisse der Fruchtwasseruntersuchung bei 108 Patientinnen dar. Mit dem Ultraschall-Echogramm gelingt die Lokalisation der Plazenta und der Lage des Kindes auf unschädliche Weise. Bei 11 Schwangeren wurde die Indikation zur intrauterinen Transfusion gestellt, die ein- oder mehrmals durchgeführt wurde. Von den Kindern überlebten lediglich 4.

Ein ausführliches Literaturverzeichnis dokumentiert die Ausführungen. Die Arbeit erweitert die bereits bestehenden Erfahrungen, bringt jedoch keine Neuerungen. Sie kann interessierten Pädiatern und Gynäkologen von einem gewissen Nutzen sein.

W. H. Hitzig, *Zürich*

M. K. Jensen: **Chromosome Studies in Acute Leukaemia.** Munksgaard, Copenhagen 1969. Preis Dkr. 50.00.

Im grossen und ganzen kann diese Monographie sowohl dem Hämatologen, der sich über den Stand (bis 1966) der chromosomalen Befunde bei akuten Leukämien informieren will, als auch dem Zytogenetiker, der sich in seinem Gebiet hämatologisch informieren will, empfohlen werden. Es werden die gebräuchlichen Techniken der Chromosomenpräparation aus Blut und Knochenmark kurz beschrieben. Zwei Kapitel befassen sich ausführlich mit den Chromosomenbefunden in Blut und Knochenmark bei akuten Leukämien. In weiteren [illegible] von Chromosomenanomalien diskutiert. Etwas spekulativ scheinen die Schlüsse, die der Autor aus dem eventuellen Betroffensein nicht nur der weissen, aber auch der erythroiden Vorläufer der leukämischen Zelle zieht. Überzeugender sind seine Hinweise auf die Veränderungen der Chromatinstruktur in den leukämischen Zellen, die dafür sprechen, dass auch in Fällen ohne abnorme Stammzellinie eine leukämische Zellpopulation vorhanden ist. Erwähnenswert sind die sehr schönen Abbildungen, die auch dem Nicht-Fachmann eindrücklich die diskutierten Phänomene wie «blurring» der Chromosomen in der leukämischen Zellpopulation, «centromere spreading», Chromatinbrücken, Chromosomenbrüche und Austauschfiguren illustrieren. Die Beschreibung der Fälle und die Tabellen am Ende der Arbeit stellen eine anschauliche Illustration der im Text dargestellten Fakten dar. Am Rande sei bemerkt, dass der Ausdruck «medial» oder «submedial» für die Centromerposition falsch ist. Es muss «median» und «submedian» heissen (Denver, London, Chicago Conferences). «Medial» ist ebenso wie «lateral» ein relativer Begriff, der die Angabe eines Bezugsobjektes verlangt.

Erika Bühler, *Basel*

H. Haefliger: **Zur Geschichte der Hämophilie unter besonderer Berücksichtigung der Schweiz.** Vol. 28. Baseler Veröffentlichungen zur Geschichte der Medizin und der Biologie. Schwabe, Basel 1969. 99 p. Preis sFr. 18.

Die Geschichte der Hämophilie eignet sich, um die besondere Leistung der Schweizer Forschung zu berücksichtigen. Die sehr grosse und gut bekannte Tenna-Familie hat viel

gen der s ch um die Elektronenm krosk spie des Knochen iarkes und des per pl eren B utes ber Fi. I. Gatenmann Happertal F'berfeld

Ol. Salmon and J. Andresen: Modern Views on the ABO Blood Groups and Secretor Status. Series Haematologica vol 2 1 Munksgaard Copenhagen 1969 Price 54 D kr

In the first article Salmon presents a tentative approach to variations in ABH and associated erythrocyte antigens. The paper comprises a review of the recent literature as well as results of his own investigations performed by means of quantitative and thermodynamic methods applied on agglutinations. The study deals first with blood group antigen modifications due to gene interactions whether allelic (weakening of A_1 by the B-gen) or not (A_m). Then the blood group modifications deficiency of A, AB, H, increase of an antigen as observed in leukaemia and refractory anaemia the appearance of red cell populations with a weakened A-antigen in elderly and some rare cases of reversible modifications in healthy subjects are treated. The possible mechanisms of the lesion of the genetic function initiating these modifications is discussed extensively.

The second article by Andresen deals with some aspects of the action of secretor gens within the ABO- and the Lewis blood group system. The serological relationship between the ABO- as well as the Lewis-antigens on the red cells and in the secretor are dealt with comprehensively. Quantitative aspects of the distribution of blood group substances in tissues and in secretions and their genetical implication are also considered. Finally aspects of the chemical nature of the blood group specific substances are included in the discussion. It is shown that recent studies fully confirm the predominant role of the Se- se-gens (besides the Le- le- Hh and ABO-gens) for the genetic control of the biosynthesis of A-, B-, H-, Le a-, Le b-substances.

Both articles, written by experts in the field, give an excellent survey of the present knowledge concerning the respective problems. R. Butler, Bern

Second Conference on the Problems of Cooley's Anemia. Consulting editor: H. Fink. Annals of the New York Academy of Sciences vol. 165 1 1969 508 p. US $ 20

In thalassemia the underlying disorder is an unbalanced hemoglobin synthesis. By a still unknown genetically determined mechanism the production of one polypeptide chain type is decreased or suppressed. Following the 12th International Congress of Hematology in New York the second conference on the problems of Cooley's anemia was held by the New York Academy of Sciences on September 9th-11th 1968. The whole series of papers is now being published in volume 165 1 of the Annals of the New York Academy of Sciences. The conference is divided in 5 parts: thalassemic syndromes and experimental models, iron metabolism, hemoglobin synthesis, genetics and therapy. In 46 papers all the different aspects of the thalassemia syndromes are discussed by the world's most competent authors. The book therefore provides an excellent review and contains the present-day knowledge of solved and unsolved problems of this widely distributed hereditary disorder. It can be recommended to the clinical hematologist as well as to the laboratory investigator of hemoglobin synthesis. H. R. Marti, Aarau

S. Witte und R. K. Zahn: Experimentelle und klinische Erfahrungen mit Cytosin-Arabinosid bei soliden Tumoren und Hämoblastosen. Arzneimittelforschung 19 Beiheft Editio Cantor 1969 19 p 98 fig 31 tab DM 24

This volume contains 22 papers presented during a recent symposium in Munich Germany about clinical and experimental experience with a new antitumor agent: the pyrimidine analogue Cytosine Arabinoside (Ara C). The meeting was organized by the German producer for Ara C (Mack, Illertissen) and relates mainly clinical experience with this particular compound. Six interesting communications present data on DNA and RNA synthesis with Ara-C treatment, studies of cellular pharmacology of the new antimetabolite and on induction of thymidine kinase and other enzyme systems by Ara C. The clinical part of 16

sehr gut wieder 1 Anti Cancer Agents Their Detection by Screening Tests and their Mechanism of Action (T A CONNORS, London), 2 Extracellular Factors Affecting the Response of Tumours to Chemotherapeutic Agents (J A DOUBLE London) 3 Intracellular Factors Influencing the Response of Tumours to Chemotherapeutic Agents (C R BALL, London), 4 Chemotherapy and Immune Reactions (J L AMIEL, Paris), 5 Dose Schedules and Modes of Administration of Chemotherapeutic Agents in Man (Y KENIS, Brüssel) 6 The Methodology of Controlled Clinical Trials (D SCHWARTZ, Paris), 7 Biological Basis of Hormonal Therapy of Cancer (H J TAGNON Brussel), 8 Operational Research in Cancer Chemotherapy Chemotherapy in the Strategy of Cancer Treatment (G MATHÉ Paris) Nur die Magistralvorlesungen des Symposiums welche ausschliesslich Spezialisten ihres Faches übertragen wurden sind abgedruckt Die Garantie erfahrener Analyse des Standes des gegenwärtigen Wissens und der Vermeidung von thematischen Überschneidungen oder Überbewertung von ins Uferlose führenden subspezialisierten Details ist daher gegeben Auch wird das viel fiszettierte Gebiet der onkologischen Chemotherapie niemals in monomaner Schematik behandelt, sondern die individuelle Betrachtungs- und Darstellungsweise jedes einzelnen Autors wird dem komplexen Sachgebiet in jeder Beziehung gerecht Langweilige Passagen fehlen Jede zusammenfassende Darstellungsweise wird erst durch eine gute Dokumentation sinnvoll Dieser Anforderung wird das Buch durch seine reiche graphische Illustration und durch präzise Literaturhinweise vollauf gerecht

Die «Recent Results in Cancer Research»-Serie wird damit um ein weiteres ausgezeichnetes Exemplar erweitert Dem onkologischen Anfänger ist es ein kompetenter Wegweiser, dem Onkologen selbst eine grosse Hilfe rasch das Wesentliche seines Fachgebietes zur Hand zu haben G A NAGEL *Basel*

D HUHN and W STICH **Fine Structure of Blood and Bone Marrow.** Lehmanns Verlag 1969 136 p, 130 fig DM 62 -

Blutzellen sind dankbare Objekte für die Elektronenmikroskopie und deshalb früh mit dieser Methode bearbeitet worden Das vorliegende Buch ist daher nicht das erste seiner Art und muss an anderen ähnlichen Ausgaben gemessen werden Es schneidet nicht schlecht dabei ab. Dazu hilft zunächst einmal die Beschränkung auf das normale Bild wie sie hier vorgenommen worden ist Weder krankhafte Zellveränderungen wie Alterungsprozesse oder Phänomene der physiologischen Zellmauserung werden berücksichtigt noch sind pathologische Zellen oder pathophysiologische Zustände bei Bluterkrankungen (Leukämien Erythrozytenanomalien primäre Knochenmarkserkrankungen usw) einbezogen Den Blutkrankheiten soll ein späterer Band gewidmet werden Man muss das wissen ehe man das Buch kauft Auch findet sich nichts über die lymphatischen Gewebe von den Lymphozyten des peripheren Blutes abgesehen Der Methodikteil stellt nur die hier angewandten Verfahren dar Jeweils von einem kurzen aber prägnanten Text eingeleitet gliedert sich der Band in zwei Teile 1 Knochenmark 2 peripheres Blut In beiden Teilen werden die normalen Zellformen beschrieben und vor allem auch reichhaltig abgebildet wobei verschiedene Nachvergrösserungen auf Details wie etwa der Zytoplasmalamellen oder der Zentriolen (z B in Plasmazellen) besonders aufmerksam machen Auf Vollständigkeit mußte wohl mit Rucksicht auf den angestrebten Umfang des Bandes verzichtet werden Um so dankbarer findet man Abbildungen der Myeloblastenfibrillen oder von zwei verschiedenen Lymphozytentypen, «dunklen» und «hellen» Lymphozyten Vielleicht wären um bei den letztgenannten Zellformen zu bleiben die Lymphozyten des Knochenmarkes auch nicht ganz zu übersehende Objekte gewesen Die Bildwiedergabe der offenbar guten primären Klischees hat durch das Papier gelitten und manche Einzelheiten wie etwa die der Leukozytengranula sind nicht deutlich zu erkennen. Das mindert aber den orientierenden Wert des Buches nur wenig Der Band gehört in die Hand jedes Morpholo-

gen, der sich um die Elektronenmikroskopie des Knochenmarkes und des peripheren Blutes bemüht.
E. Gruenbaum, Wuppertal, Elberfeld

O. Salmon and J. Andersen: **Modern Views on the ABO Blood Groups and Secretor Status.** Series Haematologica, vol. 2, 1. Munksgaard, Copenhagen 1969. Price: 34 D.kr.

In the first article Salmon presents a tentative approach to variations in ABH and associated erythrocyte antigens. The paper comprises a review of the recent literature as well as results of his own investigations performed by means of quantitative and thermodynamic methods applied on agglutinations. The study deals first with blood group antigen modifications due to gene interactions whether allelic (weakening of A_2 by the B-gene) or not (A_m). Then the blood group modifications: deficiency of A_1, A, B, H, increase of i-antigen, as observed in leukaemia and refractory anaemia, the appearance of red cell populations with a weakened A antigen in elderly and some rare cases of reversible modifications in healthy subjects are treated. The possible mechanisms of the lesion of the genetic function initiating these modifications is discussed extensively.
The second article by Andersen deals with some aspects of the action of secretor genes with the ABO- and the Lewis blood group system. The serological relationship between the ABO- as well as the Lewis-antigens on the red cells and in the secretor are dealt with comprehensively. Quantitative aspects of the distribution of blood group substances in tissues and in secretions and their genetical implication are also considered. Finally, aspects of the chemical nature of the blood group specific substances are included in the discussion. It is shown that recent studies fully confirm the predominant role of the Se-, se-genes (besides the Le-, le-, Hh- and ABO-genes) for the genetic control of the biosynthesis of A-, B-, H-, Le a- Le b-substances.

Both articles, written by experts in the field, give an excellent survey of the present knowledge concerning the respective problems.
R. Butler, Bern

Second Conference on the Problems of Cooley's Anemia. Consulting editor: H. Fink. Annals of the New York Academy of Sciences, vol. 165, 1, 1969. 500 p. US $ 20.–

In thalassemia the underlying disorder is an unbalanced hemoglobin synthesis. By a still unknown genetically determined mechanism the production of one polypeptide chain type is decreased or suppressed. Following the 12th International Congress of Hematology in New York the second conference on the problems of Cooley's anemia was held by the New York Academy of Sciences on September 9th–11th 1968. The whole series of papers is now being published in volume 165, 1 of the Annals of the New York Academy of Sciences. The conference is divided in 5 parts: thalassemic syndromes and experimental models, iron metabolism, hemoglobin synthesis, genetics and therapy. In 46 papers all the different aspects of the thalassemia syndromes are discussed by the world's most competent authors. The book therefore provides an excellent review and contains the present-day knowledge of solved and unsolved problems of this widely distributed hereditary disorder. It can be recommended to the clinical hematologist as well as to the laboratory investigator of hemoglobin synthesis.
H. R. Marti, Aarau

S. Wette und R. K. Zahn: **Experimentelle und klinische Erfahrungen mit Cytosin-Arabinosid bei soliden Tumoren und Hämoblastosen.** Arzneimittelforschung, 19. Beiheft. Ed. Editio Cantor, 1969. 192 p., 93 fig., 31 tab. DM 24.

This volume contains 22 papers presented during a recent symposion in Munich, Germany, about clinical and experimental experience with a new antitumor agent, the pyrimidine analogue Cytosine Arabinoside (Ara C). The meeting was organized by the German producer for Ara-C (Mack, Illertissen) and relates mainly to ...
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Presentations relates recent experience with the drug in the treatment of acute leukemias, polycythemia vera and certain lymphomas as well as some solid tumors. The new agent seems to be of particular interest in the attack and possibly also in the maintenance treatment of acute myeloblastic and lymphoblastic leukemias. Ara C apparently produces promising results in polycythemia vera. The drug seems to be largely ineffective in lymphomas and solid tumors. The duration of treatment courses of Ara C were usually much shorter and doses given lower than those effected by large American Chemotherapy Groups whose results are mentioned only peripherally during the discussion part. Several well documented papers deal with the hematologic toxicity of the compound especially with the profound megaloblastic bone marrow changes and with thrombocytopenia. Experimental and clinical evidence for the effectiveness of oral Ara C treatment are presented by GOLDENBERG *et al.* Of the 22 presentations 19 are given in German and 3 in English. A complete literature survey on Ara C up to 1968 (177 references) concludes the volume, which is of interest to medical oncologists and hematologists. H. J. SENN, *Basel*

I. CHANARIN: **The Megaloblastic Anaemias.** Blackwell, London 1969. 1000 p. £ 10.0.0.

Nach einem knapp gefassten historischen Überblick wird in einem ersten, mehr als 300 Seiten umfassenden Abschnitt die Biochemie des Vitamins B_{12}, des Intrinsic Factors und der Folsäure dargestellt. Die folgenden Kapitel umfassen einerseits eine eingehende Beschreibung der morphologischen Veränderungen, anderseits eine Schilderung der verschiedenen Stoffwechselstörungen, welche im Rahmen der megaloblastären Anämien zu beobachten sind. Die zweite Hälfte des Buches ist der Klinik der megaloblastären Anämien gewidmet: Klinisches Bild, Häufigkeit, Familiarität, Kombination mit andern Krankheiten, Befunde am Magen und die Neuropathie werden ausführlich behandelt. Dann wird vor allem auch die Bedeutung der Autoimmunvorgänge diskutiert. Jeder Störung, welche einer megaloblastären Anämie zugrunde liegen kann, ist ein eigenes Kapitel gewidmet, z. B. megaloblastäre Anämie nach Gastrektomie, in der Schwangerschaft, bei Blutkrankheiten mit Steigerung der (effektiven oder ineffektiven) Erythropoese usw. Eingehend werden die megaloblastären Anämien bei Jugendlichen besprochen. Auf den letzten 50 Seiten erörtert der Autor noch die therapeutischen Probleme und gibt gleichzeitig klare Richtlinien über das Vorgehen bei der Abklärung und Behandlung der Patienten mit megaloblastärer Anämie.

Zweifellos handelt es sich um eine der umfassendsten Darstellungen, die in letzter Zeit über ein grosses und wichtiges Kapitel der Hämatologie erschienen sind. Der Reichtum der Arbeit, aber auch der in den letzten Jahren erzielte Fortschritt werden deutlich, wenn man den vorliegenden fast 1000 Seiten umfassenden Band mit der seinerzeit massgebenden, knapp 200 Seiten starken Monographie von HERBERT (1959) vergleicht. Die Literatur, sowohl die ältere wie die neueste, ist sorgfältig gesichtet und verarbeitet. Die eigenen Arbeiten – sie betreffen vor allem Folsäure und Folsäuremangel – sind organisch in das Ganze eingebaut. Die Ausführungen sind immer kritisch und zurückhaltend. Als besonders gelungen dürfen die Abschnitte über Vitamin B_{12} Bindung und über den Folsäurestoffwechsel aber auch jene über die Autoimmunvorgänge bezeichnet werden. Selbst der erfahrene Hämatologe wird das Kapitel über die morphologischen Veränderungen in Blut und Knochenmark oder die Bemerkungen zur Nomenklatur mit Gewinn lesen. Eine klare Gliederung des Buches und der einzelnen Abschnitte erleichtert die Lektüre sehr. Die zahlreichen hervorragend ausgewählten und zusammengestellten Abbildungen und Tabellen tragen das ihre zum Verständnis bei. Ein flüssiger, klarer Stil macht schliesslich die Lektüre zum Genuss. Jedem Kapitel ist ein reichhaltiges Literaturverzeichnis angegliedert. Ein umfangreiches, sorgfältig zusammengestelltes Sachregister erleichtert die Benutzung des Buches als Nachschlagewerk. Wer aber etwas nachschlägt, wird bald mehr und mehr lesen wollen.

U. BUCHER, *Bern*

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(B = Book reviews, Buchbesprechungen, Livres nouveaux)

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